ANTIBODY RESPONSE IN RABBITS INOCULATED WITH *LEPTOSPIRA POMONA*

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INTRODUCTION

Leptospirosis is probably the world's most widespread contemporary zoonosis. It affects man and a wide variety of animal species. The latter form potential reservoirs of the causative agent and are sources for further propagation (Van der Hoeden, 1964). Therefore, artificial immunization is considered the only practical means for control (Stalheim, 1967). The search for a safe and effective immunizing agent has continued since the cause of leptospirosis was first identified (Alston and Broom, 1958), but generally acceptable methods have not been developed for the immunization of man (Meyer, 1964) and animals (Joint WHO/FAO, 1959).

The failure of leptospiral immunizing agents to prevent the establishment of the carrier state presents a serious obstacle to the solution of a critical epidemiologic problem. This stems from the fact that a symptomatic renal carrier, shedding leptospires, constitutes the basic unit for transmission of infection between animals and from animals to man.

The reasons for the failure to develop a completely effective leptospiral vaccine are unknown. White and Simpson (1965) suggested that formalin and other inactivating agents may remove the external enveloping sheath of the leptospires and thus may destroy or remove immunogenic antigen(s). Therefore, the animals injected with such a vaccine may not produce all the necessary antibodies for protection.

The present investigation describes the nature and the sequence of antibody in the primary and secondary immune responses to both live and formalin-inactivated *Leptospira pomona* in rabbits. The sequential appearance of γM (19S) and γG (7S) immunoglobulins in the primary and secondary antibody response in rabbits injected with live and formalized cells of *L. pomona* were demonstrated. Certain serological and physicochemical properties of the antibody produced at successive intervals following immunization were also described. The effect of the administration of passive antibody on the subsequent synthesis of immunoglobulin was studied. The following points were demonstrated:

1. The primary response to both live and formalized cells of *L. pomona* included the early synthesis of 19S, γ-1 globulin antibody and the later synthesis of 7S, γ-2 globulin antibody.

2. The secondary response to the same antigens is comprised of the early synthesis of large amounts of both kinds of immunoglobulins, 19S (γM) and 7 S (γG).

3. Treatment of the macroglobulin (19S, γM, or IgM) with mercaptoethanol resulted in complete loss of the agglutinating activity.

4. An unusual feature of the immunological response, the persistence of γM for long periods of time (at least six months), was demonstrated.

5. Rabbit γM and γG anti *L. pomona* antibodies were isolated from two fractions of immune sera prepared by DEAE-cellulose chromatography and Sephadex gel filtration. The two protein preparations were examined by several immunochemical criteria, with results which justified their identification as γM and γG antibodies.

6. Passive immunization of rabbits with anti-*L. pomona* serum significantly inhibited the primary response to antigenic stimulation by leptospires.

7. The immunosuppression was specific and apparently was produced by 7S immunoglobulin.

8. The immunosuppressive effect of specific antibody on the primary response contrasted sharply with the absence of this effect on the secondary response.

LITERATURE REVIEW

Antibody Heterogeneity

Numerous reports indicate that at least two distinct molecular classes of antibodies are formed sequentially during the primary response (Bauer and Stavitsky, 1961; Benedict *et al.*, 1962; Uhr and Finkelstein, 1963; Svehag, 1964; Svehag and Mandel, 1964). The first antibody to be detected in the serum after antigenic stimulation was γM (macroglobulin, 19S, or IgM). This initial antibody usually declines or disappears completely in several weeks to be replaced by γG (7S or IgG) which may persist for long periods of time. Such sequential syntheses have been extensively studied in the rabbit and have also been reported in various animal species in response to soluble and particulate antigens of diverse composition. However, Pike and Schulze (1964), Pike *et al.* (1966), Robbins (1965), and Weidanz *et al.* (1964) observed that a secondary stimulus or hyperimmunization was required to elicit detectable amounts of γG. In the majority of cases the secondary response was composed mainly of γG.

Sequential Appearance of Immunoglobulins in Rabbits

Benedict *et al.* (1962) described the electrophoretic, ultracentrifugal, and chromatographic separation of early and late appearing antibodies in rabbits injected with bovine serum albumin (BSA) and employing the passive hemagglutination (HA) method for antibody detection. Antisera had at least two types of antibodies: one associated with a fraction characterized as γ-2 globulin had a sedimentation coefficient of 7.3 S (fraction I on diethylaminoethylcellulose), and another type associated with a fraction characterized as a γ-1 globulin had a sedimentation coefficient of 20.4 S (fraction IV on diethylaminoethylcellulose). The HA titers of fractions I and IV of early sera were approximately equal. On prolonged antigenic stimulation fraction IV decreased in concentration and the relative concentration of fraction I agglutinins increased. Bauer *et al*. (1963) determined the nature of antibodies produced in rabbits during the primary and secondary response to T_2 phage, proteins, and the O and H antigens of *Salmonella typhosa*. Immune sera were fractionated by zone electrophoresis, sucrose density gradient centrifugation, and anion exchange chromatography. With the exception of the O antigen (lipopolysaccharide) of *Salmonella*, the primary response included the early synthesis of 19S, γ-1 globulin antibody, and the later synthesis of 7S, γ-2 globulin antibody. The secondary response to the phage consisted of the synthesis of 7S, γ-2 globulin antibody alone. Moreover, the author reported that treatment of the macroglobulin phage-neutralizing antibody with mercaptoethanol resulted in complete loss of its neutralizing activity. A working hypothesis was presented to explain this sequential appearance of antibodies. A salient feature of this hypothesis was the suggestion that different types of cells synthesized the two distinct molecular forms of antibody. Grey (1964) reported that there was a sequential change in the class of rabbit-bovine serum albumin antibodies following immunization. In addition, the author related this change in binding strength of antisera to the change in the molecular size of the antibody from the fast sedimenting 19S to the more slowly sedimenting 7S molecules. The antibody response was studied by giving a single inoculation of antigen with 2 mg of alum. On the eleventh day post-inoculation (PI), the hemagglutination titer revealed a predominance of 19S antibody. On the seventeenth day PI virtually all the antibody was present in the 7S fraction. Bauer and Stavitsky (1961) presented physicochemical and serological evidence for the appearance of two distinct molecular species of antibodies. The early response to a single injection of diphtherial toxoid in rabbits consisted of the initial synthesis of γ-1 macroglobulin antibody followed by the synthesis of γ-2, 7S globulin antibody. The γ-1 macroglobulin antibody lost specific hemagglutinating and precipitating activity upon treatment with 2-mercaptoethanol, but the 7S antibody did not lose its activities. The γ-1 macroglobulin seemed to be inactivated by heat at a lower temperature than the γ-2 globulin antibody.

Dixon *et al.* (1966) studied the antibody response of rabbits and rats to hemocyanin, the copper-containing respiratory protein of mollusks and arthropods. The authors reported that in all animals tested, the first antibody to appear was entirely mercaptoethanol sensitive (MES) and within one to two weeks the amounts of MES and MER (mercaptoethanol resistant) antibody were nearly equal for all antigen doses. In addition, this balance of MES and MER antibodies appeared to be maintained throughout the primary response with no indication that the advent of MER antibody formation limited or terminated MES antibody synthesis. The secondary response was characterized by higher circulating antibody levels, four to seven times those of the primary response. The observation that one-fourth to one-fifth of the antibody throughout the secondary response was MES, was in contradiction to the observations of Bauer and Stavitsky (1961), Svehag and Mandel (1964), Uhr and Finkelstein (1963) and LoSpalluto *et al.* (1962). However, Dixon concluded that in anti-keyhole limpet hemocyanin (anti-KLH) responses, sizeable amounts of MES and MER antibody synthesis appeared to coexist for long periods of time. Furthermore, Bauer (1963) attempted to determine whether antibodies to the haptene were associated with the same globulins as the antibodies to the antigen. Rabbit hemagglutinating antibodies to sulfanilic acid and p-aminobenzoic acid were found to be heterogeneous with respect to their molecular size. Studies on the anti-haptene globulins indicated that the antibody activity was of both the γM and γG types. After an initial series of antigen injections, the anti-haptene activity was predominantly the macroglobulin type. With continued immunization, the antibody activity shifted to the 7S type.

Stelos and Taliaferro (1957) described the separation, using starch gel electrophoresis, of the two antibodies in rabbit antiserum to the Forssman and isophile antigens of sheep red blood cells (SRBC), which differed in hemolytic efficiency. With Forssman antiserum the peak of hemolytic activity was between the β and γ globulins (referred to as γ-1 globulin), whereas the peak combinining activity was in the more slowly moving globulins (referred to as γ-2 globulins). With isophile antisera the peaks of hemolytic and combining activity were not as widely separated as with Forssman antisera. Peak hemolytic activity was near the midpoint of the γ globulin fraction and peak combining activity was found, as in the case of Forssman antisera, in the γ_2 globulin. Moreover, it was found that in animals given a few injections and bled after a short period of immunization, most of the hemolytic antibody produced was of the type of γ_1 fraction. At this time, however, antibody was also found in the γ_2 fraction. It was only after a lengthened period of immunization, that the antibody level in the γ_2 fraction had considerably increased. This shift in relative amounts of antibody in γ_1 and γ_2 fractions was inconsistent with previous findings (Talmage *et al.*, 1956).

Pike and Schulze (1961) studied the distribution of antibodies against erythrocytes in chromatographic fractions of early and late immune sera. The sera of rabbits injected intravenously with sheep, human O, and bovine erythrocytes respectively were collected at various stages during immunization and were fractionated on a DEAE-cellulose column. In early sera, against all three types of cells, the predominant agglutinating activity was associated with fractions containing macroglobulins (γM) eluted by phosphate buffer of higher molarity. On continued immunization, however, agglutinins appeared in fractions eluted from the cellulose by phosphate solutions of lower molarity. It was also found that the later agglutinins sedimented more slowly on ultracentrifugation than the former. In addition, the failure of early agglutinins to react with the rheumatoid factor was shown to be due to the fact that the agglutinins appearing first were macroglobulins which were not reactive. Bauer *et al.* (1963), LoSpalluto *et al.* (1962), and Nicoli *et al.* (1965) reported that the type of antibody produced following stimulation with somatic polysaccharide was exclusively macroglobulin in nature. This observation was challenged by Weidanz et al. (1964), Pike and Schulze (1964) and Pike et al. (1966), who provided clear evidence that under certain conditions rabbits could also produce 7S antibody specific for the polysaccharide complex. Moreover, it was also reported that repetitive stimuli and a large amount of antigen were required to evoke a significant amount of 7S antibody response.

Pike et al. (1965) demonstrated that the sera from patients with leptospirosis taken from the second to the fifth week after onset of disease showed an increase in the proportion of γG. This increase was determined by microscopic agglutination (MA) tests following treatment with 2-mercaptoethanol. Amatoni et al. (1958) performed a series of tests on fifteen sera from people with Weil's disease, and from one guinea pig which had been experimentally infected with Leptospira icterohaemorrhagiae. All sera were tested for agglutinins on days 10, 17, 27, and 40. Antibodies were found in the guinea pig on the fortieth day. In addition, using electrophoresis studies, it was found that the predominant antibody in human early sera was γ_1 (γM) and in late sera γ_2 (γG).

Daniel (1965) described the results of experiments undertaken to examine the sequence of molecular species of antibody produced in rabbits in response to mycobacterial antigens. The primary response consisted of the early synthesis of γM antibody followed by the production of persisting 7S γ_2 antibody. The anamnestic response was characterized by the accelerated production of large amounts of 7S antibody. The γM antibody reappeared at the same time and in the same amounts as it did after the primary injection.

In a later study on the physicochemical properties of the antibody produced at various times after immunization with poliovirus, it was reported that anantibody of high molecular weight (19S) was present in 2 to 3 day sera. On days 3 and 4 7S was also detectable. Both types increased for one to two weeks, after which 19S antibody began to decline, while 7S antibody remained almost undiminished. On the other hand, when a small dose of antigen was used, the response was simpler: only the 19S type was detectable throughout the period of observation, even when a second stimulus with a small dose was administered.

Hocker and Bauer (1965) observed the nature of antibodies synthesized during the immune response to Leptospira biflexa. It was shown that MA antibody could be detected 48 hours after initial injection. The peak primary response of MA antibody occurred on days 6 to 10. Peak MA secondary antibody titers were reached at 4 to 9 days. Examination of primary and secondary responses of antisera by density gradient ultracentrifugation and sensitivity to reduction indicated that both the primary and secondary activities were due to the synthesis of 19S antibody.

Sequential Appearance of Immunoglobulins in Mice

On the basis of density gradient centrifugation, behavior on DEAE-cellulose columns and susceptibility to mercaptoethanol treatment, it has been shown (Turner and Rowe, 1964) that the antibodies produced in mice in response to infection with virulent Salmonella typhimurium were of two distinct molecular types. The first phase of antibody production was characterized by a 19S macroglobulin response which disappeared after about one month, to leave the 7S type antibody which persisted for at least six months.

Biphasic responses and different rates of neutralizing activity of early and late sera from BALB/SY mice were demonstrated (Kim *et al.*, 1964). Mice given single and multiple injections of actinophage produced two types of antibodies as shown by phage neutralization. By using physical and immunochemical techniques, only 19S (γM) antibody could be demonstrated in the early sera. The late sera contained mostly a γ_2 immunoglobulin of the 7S class.

Adler (1965) subsequently reported on the immune response to one or more intraperitoneal injections of sheep red blood cells. Early lysins and agglutinins were ME-sensitive and those appearing later were 7S, ME-resistant γ-globulins. Mice injected once with ovalbumin or ferritin in Freund's adjuvant responded by producing 19S and 7S antibodies. Although 19S antibody production continued for a long time, the relative proportion of 7S to 19S was 5 to 1, two weeks after immunization and 150 to 1 after 11 weeks.

In the report by Kenny and Herzberg (1967) using a bactericidal assay for determining circulating antibody, it was observed that after immunization with either live or heat-killed *S. typhimurium*, mice responded with an extremely rapid (two days) production of bactericidal antibody. Inactivation of sera with mercaptoethanol, along with Sephadex fractionation, indicated that the observed bactericidal activity was associated with a macroglobulin (19S) which was completely mercaptoethanol sensitive. However, the activity associated with the later response (14 to 35 days after infection or immunization) involved the presence of γG as well as macroglobulin (19S) activity.

In the study on the immune response of mice to influenza virus, Berlin (1963) reported that hemagglutination-inhibition (HI) antibodies appeared in the serum of mice three days after injection of influenza virus vaccine. The initial antibody (18S) was sensitive to 2-mercaptoethanol and had a biologic half life of 0.5 days. Antisera obtained two weeks after vaccination contained 7S immunoglobulin that resisted treatment with mercaptoethanol and which had a half-life of two days.

Furthermore, antibody response in rate to primary injection of flagella (Ada *et al.*, 1965) was characterized by a lag period of three days followed by a sharp rise in antibody titer over a 3 to 4 day period. Most of the antibody formed in the first week was macroglobulin. Antibody present at three weeks and thereafter was 7S.

Sequential Appearance of Immunoglobulins in Guinea Pigs

In reports by Brown *et al.* (1965) and Cowan and Trautman (1965) on studies of the early antibody in the sera of guinea pigs infected with foot-and-mouth disease virus, it was observed that the neutralizing activity of seven day serum sedimented in a sucrose gradient much more rapidly than 7S γ-globulin. This early viral antibody was found to be 19S γ-globulin. Moreover, the neutralizing activity of early sera was completely destroyed by 2-mercaptoethanol (2ME), whereas the antibodies present later (21 days) after infection were unaffected by mercaptoethanol. In a subsequent report, Graves *et al.* (1964) characterized the antibodies produced by guinea pigs inoculated with inactivated foot-and-mouth disease virus (FMDV). He was able to demonstrate two types of neutralizing antibodies with markedly different physicochemical and complement fixing properties. One type present during the first and second week after inoculation, was of the 19S class, had a high adsorption to DEAE-cellulose, and was unable to fix complement. The other type appeared during the second week after inoculation and was of the 7S class, was poorly adsorbed on DEAE-cellulose, and was able to fix complement.

In a study of anti-ϕX 174 formation in guinea pigs certain similarities between the kinetics of the primary 19S and the secondary 7S antibody formation were indicated (Uhr and Finkelstein, 1963). Later, the same investigators determined the decline of 19S antibody formation, the primary 7S antibody response, and the relationship between the primary and secondary responses. It was observed that the primary 7S antibody formation lasted for months and led to preparation for a secondary 7S antibody response (immunological memory). In contrast, 19S antibody formation was relatively short-lived and did not lead to immunological memory.

In a study following experimental infection of guinea pigs with Japanese encephalitis and Russian spring-summer encephalitis virus (Bellanti *et al.*, 1965), complement fixing (CF), hemagglutination inhibition (HI), and neutralizing (N) antibodies were regularly formed by the seventh to the tenth days. Characterization of these antibodies at various times over a four month period revealed the CF antibody was invariably associated with a 7S γ_2-globulin. There was no evidence of an early 19S complement-fixing antibody. The earliest detectable HI and N antibodies at 7 to 10 days were largely associated with a 19S γ-globulin. After 14 days, these activities were found in the 7S γ-globulin.

Sequential Appearance of Immunoglobulins in Humans

There have been few opportunities to observe the sequence of antibody appearance in man. These have been in naturally occurring infections. Murray *et al.* (1965a, b) have shown that the antibody in human primary typhus was predominantly γM, whereas in recrudescent disease (Brill Zinsser disease) it was γG. In human volunteers (Reddin *et al.*, 1965) experimentally infected with Brucella, the early agglutinin was γM; γG was not observed until about two weeks after infection. LoSpalluto *et al.* (1962) reported that neonatal infants, older children, and adult humans produced 19S and little or no 7S antibody after initial immunization with typhoid-paratyphoid antigens. In premature infants the change from 19S to 7S antibody in response to paratyphoid A and B was virtually complete at 13 weeks of age, whereas the change in typhoid H agglutinins from 19S to 7S was slower. In the adult the change in typhoid H and paratyphoid A and B agglutinins from 19S to 7S required more than six months. Booster injections of these organisms resulted in the production of 7S antibody exclusively. Furthermore, typhoid O agglutinins were found only in 19S fraction in all of these groups, and no change accurred with time or with secondary immunization. In a subsequent study (Turner and Rowe, 1964) on the appearance of antibodies to H and O antigens in humans after immunization with a heat-killed typhoid vaccine showed that serum antibodies to the H antigen were initially of the γ_1M class. Later the γG class became predominant. In contrast, serum antibodies to the O antigen were of the γ_1M class only.

Sequential Appearance of Immunoglobulins in Cattle

In studies describing the sequential appearance of immunoglobulins in cattle, Rose and Pompke (1964) demonstrated that, as a result of *Brucella* infection, the early agglutinin was in the γ_1M class; 7G was not observed until two weeks after infection. Similar findings were obtained with sera from cattle at 7, 14, and 21 days after infection with foot-mouth-disease virus. With these sera, the major part of the virus neutralizing activity (7 day) was completely destroyed with 2-mercaptoethanol treatment. In contrast, neutralizing activity present later (21 days) in infection was unaffected by the same treatment.

Sequential Appearance of Immunoglobulins in the Chicken, Turtle, Frog, and Goldfish

Uhr *et al.* (1962) showed that after a single injection of 10^8 or 10^9 bacteriophage (ϕX 174), the chicken, frog, and goldfish produced approximately the same level of neutralizing, rapidly sedimenting, γ-globulin antibodies. Repeated injection of bacteriophage at intervals of 2 to 4 weeks, did not elicit an anamnestic antibody response. However, higher levels of antibody, mainly in the slowly sedimenting γ-globulin fraction, were produced after immunization with bacteriophage in complete Freund's adjuvant. On the basis of these findings, the authors concluded that this change was similar to the replacement of 19S by 7S antibodies in the circulation of the immunized mammals. Later, chicken anti-BSA antibodies formed following primary, secondary and tertiary antigenic stimulation were characterized (Benedict *et al.*, 1963). Evidence was presented that the sequential synthesis in the chicken of HA antibody against BSA was similar to rabbit antibody in that high concentrations of chromatographically distinct macroglobulin (19S) were synthesized initially.

In the work reported by Riha (1965), it was shown that antibodies, after immunization of chickens with haptene, were of the 19S type. Repeated antigenic stimulation was required to elicit detectable amounts of 7S γ-globulin. In a recent investigation Rosenquist and Campbell (1966) attempted to characterize the 19S and 7S response to bacteriophage ϕX 174 in the fowl. It was shown that Sephadex peak I (19S globulin) accounted for most of the early activity of the primary response. By day 9 most of the activity had shifted to peak II (7S globulin). In the secondary response, the shift had occurred by the fourth day. Moreover, the authors reported that all serum fractions (whether 19S or 7S) were sensitive to reduction with 2-mercaptoethanol.

Studies by Grey (1963) on the immune response in the turtle showed that the early γM response was prolonged compared to that observed in mammals. The prolonged heavy antibody response in the turtle appeared to be the major molecular species of antibody for 30 to 40 days following immunization and was still present in lesser amounts for at least four months following initial immunization. However, some recent observations suggested that the sequential synthesis of γM and γG could be more apparent than real. Freeman and Stavitsky (1965), using radioimmunoelectrophoresis, detected the simultaneous appearance of γG and γM antibodies in rabbits after the injection of human serum albumin (HSA) and bovine γ-globulin. Using a method which measured the antigen-binding capacity of rabbit antibodies for HSA, Osler *et al.* (1966) found even greater quantities of γG than of γM within the first week after antigen injection. Cushing and Johnson (1966) presented evidence that a γG antibody represented a significant portion of the early antibody response in mice to HSA. Also, Cohen *et al.* (1967) showed that a significant portion of natural human antibody to Gram-negative bacteria resides in the γG class of immunoglobulins when these natural antibodies were assayed by the indirect fluorescent antibody (IFA) technique. It became clear that the type of immunoglobulins detected was dependent upon the assay method employed, since the amount of antibody required to give a positive reaction in the same assay method differs depending upon the class of immunoglobulin being studied; i.e. the complement fixation test (CF) required 1 to 2 molecules of γM antibody but nearly 2000 molecules of γG antibody to give a detectable positive reaction. Therefore, various assay methods to measure a specific class of immunoglobulins must be taken into account in the interpretation of the results of the immunologic tests.

Studies on the Influence of Antibody on the Immunogenic Response

It has long been recognized that pre-existing antibody, either endogenously formed or passively administered near the time of the injection of the corresponding antigen, can affect the subsequent antibody response. An excess of antibody, or in some cases, even an equivalent amount of passively administered antibody can partially or completely inhibit the primary response in a previously unchallenged animal.

Smith (1909) showed that an active immunity lasting several years can be produced in guinea pigs by the injection of toxin-antitoxin mixtures which have no recognizable harmful affect, either immediate or long-range. It was also shown that under the same conditions, mixtures which produce local lesions and which, therefore, contain an excess of toxin produced a much higher degree of immunity than the neutral mixtures, and that an excess of antitoxin reduced the possibility of producing an active immunity. On the basis of these findings, Smith concluded that there was a definite relation between the components of the mixture and the degree of producible immunity. This observation was subsequently confirmed (Glenny and Sudmersen, 1921; Barr *et al.*, 1950; di Sant Agnese, 1949; Osborn *et al.*, 1952). In a later study groups of guinea pigs were passively immunized (Mason *et al.*, 1955) against diphtheria toxin with homologous antitoxic serum so that these sera contained, at the start of the experiment, 0.1, 0.01 or 0.001 unit per milliliter, respectively. They were then actively immunized with one or two injections of 0.1 Lf of adsorbed dissolved flocculates (ADF). Passively introduced serum which had titers of 0.01 and 0.001 unit per milliliter did not interfere with active immunization in any demonstrable way. However, a titer of 0.1 unit per milliliter did markedly interfere with active immunization four weeks after the primary injection and nearly two weeks after the secondary stimulus. The highest serum titer was obtained at the thirty-second week instead of at the fourth week, as in the guinea pigs actively immunized. The above observations have been extended to the study of additional antigens, such as tetanus (Buxton and Glenny, 1921; Otto and Hennemann, 1939; Regamey and Aegerter, 1951), SRBC's (Talmage et al., 1956), poliomyelitis virus (Rhoads, 1931), and bacteriophage (Kalmanson and Bronfenbrenner, 1943).

Uhr and Baumann (1961) demonstrated that the suppression of antibody formation by passively administered antibody was influenced by the dose and nature of the antigen, the type of immunization procedure, the ratio of antibody to antigen, the species origin and characteristics of the antiserum used, as well as the species selected for immunization. Evidence was presented that in guinea pigs, diphtheria antitoxin formation can be effectively suppressed by an intravenous injection of excess homologous or heterologous antitoxin as long as five days after toxoid immunization or after delayed hypersensitivity to toxoid has developed. Following the period of antibody suppression, which lasted 2 to 7 weeks, serum antibody could usually be demonstrated.

Neiders et al. (1962) reported that when adult rats were immunized with sheep erythrocytes, the secondary hemolysin response was lower than the primary response. The effect of circulating hemolysin on the rat antibody response to sheep erythrocytes was also investigated. A marked inhibition of the primary hemolysin response in the rat was produced by administering homologous anti-SRBC serum 24 hours before the injection of sheep erythrocytes. Inhibition of the antibody response by antiserum occurred when rats were actively immunized intravenously, intraperitoneally, or intradermally. The presence of circulating hemolysin was necessary to demonstrate the inhibition of the hemolysin response. Subsequently, Rowley and Fitch (1964) reported that passive immunization of rats with homologous anti-SRBC serum markedly inhibited the primary antibody to various doses of sheep erythrocytes. Inhibition was specific and apparently was produced by either 19S or 7S antibody. Also, passive immunization inhibited splenic hyperplasia associated with primary antibody response. The distinct suppressive effect of splenic antibody on the primary response contrasted sharply with the absence of this effect on the secondary response. Moreover, exposure of normal spleen cells to the antibody in vivo or in vitro suppressed their response to the antigen in the X-irradiated recipient. Accordingly, it was concluded that the formation of specific antibody may provide a homeostatic mechanism which controls or limits production of specific antibody to the antibody-forming system which was stimulated previously by the antigen. Möller (1964) reported that antibody treatment of lymph cells did not inhibit their capacity to give an antibody response and concluded that the immune suppression induced by antibodies injected subsequent to the antigen did not necessarily indicate that a central step in the active antibody response was suppressed. In the mouse (Herzenberg et al., 1967) it was found that antibody directed against an immunoglobulin allotype, Ig-1b marker, passed from mother to offspring, or injected into neonates, suppressed synthesis of immunoglobulin carrying the Ig-1b marker. In allotype homozygotes as well as heterozygotes the allotype suppression was manifested both by a delay of several weeks in attaining initial detectable allotype levels and a reduction in allotype level which continued into adulthood.

Finkelstein and Uhr (1964) reported on the comparative effect of passive administration of 19S and 7S immunoglobulins on the inhibition of antibody formation. They found that when 7S immunoglobulin was administered ten days before immunization, either 19S nor 7S antibody formation, nor immunological memory to φX 174 developed. In contrast, similar administration of 19S antibody did not affect any of these three aspects of the subsequent immune response to φX 174. In addition, these authors observed that secondary 7S antibodies differed markedly in their capacities to inhibit antibody formation, that 7S antibody injected at the time of immunization rather than 2 to 3 days later inhibits 19S formation more effectively and that 19S antibody did not influence 7S formation but could depress 19S formation. It was obvious that the 19S immunoglobulin was considerably less effective than the 7S immunoglobulin in the inhibition of antibody formation to φX 174. In addition, Finkelstein and Uhr (1964) speculated that 7S antibody interferes with the extracirculatory transport of antigen which, they postulated, occurred around the third day.

In a subsequent report by Sahiar and Schwartz (1964) it was shown that injection of 6-mercaptopurine into the animals prevented the synthesis of 7S antibodies during the immune response of rabbits to bovine gamma globulin and did not affect the γM response. Specific reactive homologous 7S antibodies given intravenously to animals with such incompletely developed humoral reactions led to an abrupt fall in 19S antibodies. After a lag period the synthesis of 7S antibodies was apparently normal. This demonstrated that the concentration of 7S antibodies controls, at least partially, the synthesis of 19S antibodies.

Moller and Wigzell (1965) studied the suppressing activity of passively transferred antibodies on antibody synthesis against sheep red blood cells at the cellular level. The Jerne method was employed (Jerne and Nordin, 1963). Humoral antibody injected prior to the antigen suppressed completely the appearance of plaque-forming spleen cells producing 19S antibodies. The inhibiting efficiency of 7S antibodies was about 100 to 200 times greater than that of 19S antibodies. The authors suggested that humoral antibodies inhibit the immune response by removing the stimulus for the proliferation of the antibody-producing cells and not by directly depressing antibody synthesis in already committed cells. The above finding was confirmed by Wigzell (1966). The technique of direct and indirect hemolytic plaque tests was used to investigate the specific supressing activity of passively administered antibody on 7S antibody synthesis against sheep and chicken red blood cells. The 7S production was found to be sensitive to antibody-induced suppression. Moreover, no inhibitory effect of transferred antibody was seen until 48 to 70 hours after administration. This indicated that the action of antibody was not by direct suppression of synthesis of already committed cells but rather by removal from the system of the stimulus for maintenance of 7S synthesis. This work also emphasized the role of antibody as feedback factor during a substantial post-peak period of 7S antibody synthesis and suggested an important role of antigen in stabilizing the 7S antibody production.

A recent report by Dixon et al. (1967) on the effect of passively administered antibody on antibody synthesis, showed that suppression of the primary response of rabbits to intravenously administered KLH could be achieved with a very small amount of hyperimmune anti-KLH administered a day later. The amount of anti-KLH needed to achieve immunosuppression was directly proportional to the dose of injected antigen. Furthermore, antibody passively administered as much as 6 to 8 days after antigen still could be strongly immunosuppressive, which suggested that the antibody must be reacting with an immunogenic substance in or on responding cells or might be reacting in the process of transfer between cells. In addition, the workers presented some evidence which suggested that passive antibody neutralizes the immunogenic stimulus at the level of individual antigenic determinants.

Taken together, the various findings suggested that antibody suppression represents a major feedback mechanism controlling the number of antibody-producing cells against antigens, which persisted in an immunogenic form for long periods of time. According to this concept, it would be expected that stimulation of antibody production in an environment lacking suppressive antibodies would lead to excessive antibody synthesis and to an uninhibited multiplication of the committed cells. This hypothesis was tested (Möller, 1968) by transfer of spleen cells from mice immunized against SRBC's into irradiated (600R) nonimmune mice in the presence of antigen. This experiment resulted in excessive cellular 7S production seven days later. The number of 7S plaque-forming cells (PFC) usually exceeded 10^6 per spleen. Serum antibody synthesis was also excessively increased. These results suggested that the excessive production of 7S (PFC) and antibodies in the irradiated nonimmune recipients was caused by the absence of feedback inhibition of the immune response by antibody, a mechanism which would normally function to restrict antibody synthesis.

The above concept was challenged by another group of workers headed by Jerne (1955) who reported that the rate of antibody production after a primary stimulus depended on the concentration of spontaneous antibody already present in the circulation. The response to an antigen of an animal whose serum contained a low level of specific antibody was greater than that of animals in which no antibody could be demonstrated. Antibody introduced from an immunized donor into the blood of a recipient prior to the injection of antigen into the latter enhanced antibody response to this antigenic stimulus. Segre and Kaeberle (1962) observed that colostrum-deprived baby pigs lacked a factor (S) contained in colostrum which participates in the process of antibody production. They also showed that a small amount of specific antibody administered with the antigen replaced the colostrum factor and provided sufficient stimulus for antibody production. It was also found that large amounts of serum from nine-week-old, colostrum-deprived pigs inoculated in conjunction with antigen had a moderate stimulatory effect on antibody production. In a later study, Hoerlein (1956) reported that colostrum-deprived pigs which did not produce detectable antibody following a single injection of antigen were, nevertheless, capable of an antibody response as a result of a second inoculation with the same antigen three weeks later.

In subsequent work by Locke *et al.* (1964) it was demonstrated that intraperitoneal administration of 75 mg of crude ovine globulin, containing antibodies in both the 6.6S and 18S fractions, resulted in the production of anti-SRBC globulin by the recipient pigs. Moreover, dilute 6.6S ovine antitoxin, premixed with toxoid and injected intraperitoneally into three week-old, colostrum-deprived pigs, induced the production of relatively large quantities of tetanus and diphtheria antibodies by the recipient pigs. These observations were confirmed by finding an enhanced immunological response to bovine serum albumin (Terres and Wolins, 1959) and by the demonstration of a third tetanus toxoid in mice when these antigens were injected with specific heterologous antisera (Terres and Stoner, 1962).

In a subsequent report Möller and Wigzell (1965) reported that 19S antibody may elicit a minor enhancement when a small dose was given, but would suppress the immune response at higher doses. Further experiments (Henry and Jerne, 1967) indicated that purified 19S antibody consistently enhanced the primary response. An enhancement of the primary response was also reported after a low antibody dose (Terres and Morrison, 1967).

SUMMARY OF LITERATURE

In the above review, an attempt has been made to assemble information about the sequential appearance of the principal immunoglobulins (19S and 7S) in serum of a wide variety of animal species as well as information about the effect of passive administration of these immunoglobulins on the immunogenic response.

The heterogeneity of antibody as determined by a variety of physical, chemical, and biological methods has been well established. Numerous reports indicate that at least two distinct molecular classes of antibodies are formed in a sequential manner during the primary response (Bauer and Stavitsky, 1961; Benedict et al., 1962; Uhr et al., 1963; Svehag, 1964). The primary response to antigen is usually characterized by a transient rise in 19S antibody followed by the appearance of 7S antibody. The secondary response is usually characterized by the rapid synthesis of predominantly 7S antibody. As already indicated, there was conflicting evidence in some areas, and in others the data was subjected to different interpretations. Recent evidence suggests that 7S antibody can appear prior to, or concomitant with, the appearance of 19S antibody (Freeman and Stavitsky, 1965; Osler et al., 1966; Cushing and Johnson, 1966; Cohen et al., 1967). It is possible that conflicting findings are due to the differences in methods used to assay for the activity of 7S and 19S antibodies.

In addition, it has been suggested that antibody plays an important role in regulating immunogenic responses by specific feedback mechanisms (Uhr and Baumann, 1961; and Rowley and Fitch, 1964) and, further, that 7S antibody has a particular role in this regard. These reports indicated that 7S but not 19S antibody was an effective inhibitor of antibody production. Others revealed that the ability to suppress antibody formation was not a property exclusive to a particular immunoglobulin type. Both 7S and 19S antibodies were shown to be capable of suppressing the formation of antibody to significant degrees (Pearlman, 1967). An enhancement of the primary response after low antibody doses has been reported by Segre and Kaeberle (1962), Terres and Stoner (1962), Locke *et al.* (1964), and Terres and Morrison (1967). Differences in the results may relate partly to differences in methods used to assay for the activity of 7S and 19S antibodies administered or partly due to differences in methods used for separation of the two types of immunoglobulins.

This study of heterogeneity of antibody produced in response to antigenic stimulation of leptospires has been undertaken in an attempt to determine the sequential synthesis of 19S and 7S immunoglobulins. In addition, in an effort to define the nature of the influence of leptospiral antibodies on immunologic responses to leptospiral antigens, experiments were designed focusing on the effect of leptospiral immunoglobulins employed in this investigation. The methods used in separation as well as in measuring the activity of the two immunoglobulin types will be considered in the interpretation of the results.

MATERIALS AND METHODS

Media and Reagents

1. Fletcher's medium: A complex semisolid medium, Difco Laboratories, Detroit, Michigan.

2. Stuart's medium: A complex dehydrated liquid medium, Difco Laboratories, Detroit, Michigan.

3. 2-Mercaptoethanol (2ME): A reducing agent, Eastman Organic Chemicals, Kingsport, Tennessee.

4. Iodoacetamide (IA): An alkylating agent, Mann Research Laboratories, New York, New York.

5. Tris (hydroxymethyl) aminomethane (Tris): A biochemical buffer, Sigma Chemical Company, St. Louis, Missouri.

6. Barbital buffer: A biochemical buffer used in immunoelectrophoresis, Gelman Instrument Company, Ann Arbor, Michigan.

7. Barbital buffer: A biochemical buffer used in microzone electrophoresis, Beckman Instrument, Inc., Harbor Boulevard, Fullerton, California.

8. Special Noble agar. A solidifying material, Difco Laboratories, Detroit, Michigan.

9. Sodium azide: A chemical used in microbiological procedures as a bacteriocide or fungicide, Baltimore Biological Laboratory (BBL) Inc., Baltimore, Maryland.

10. Crystalized human albumin: human protein used in preparing standard solution, Dade Reagents Inc., Miami, Florida.

11. Biuret reagent: A biochemical reagent, Hycel Research Products, Hycel Inc., Houston, Texas.

12. Ponceau S: Fixative dye solution, Beckman Instruments, Inc., Harbor Boulevard, Fullerton, California.

13. Buffalo Black NBR (Naphthol blue black): A biological stain, Allied Chemical Corporation, New York, New York.

14. Sephadex G-200: A cross-linked dextran gel, Pharmacia Fine Chemicals Inc., New Market, New Jersey.

15. Diethylaminoethyl-cellulose (DEAE-cellulose): Anion exchange cellulose (Cellex-D). Cellex-D is a highly purified cellulose powder containing diethylaminoethyl exchange groups, Calbiochem, Los Angeles, California.

16. Merthiolate: A chemical used in microbiological procedures as a bacteriocide, Eli Lilly and Co., Indianapolis, Indiana.

Bacterial Strains

Two serotypes of Leptospira were employed in this investigation.

1. *Leptospira pomona* pomona.—A pathogenic Serotype
2. *Leptospira biflexa* CDC.—A saprophytic Serotype

Laboratory Animals

New Zealand white rabbits (at least three pounds) were employed in the preparation of leptospiral antisera. Prior to immunization, serum was obtained from each rabbit and was tested for agglutinating antibody. No antibody titer could be detected in the uninoculated rabbits. All animals received standard commercial laboratory feed (Rabbit Chow) and water.

Cultural Procedure

All stock cultures were maintained in Fletcher's semisolid medium which contained heat-inactivated (56 C for 30 minutes) 8 per cent hemolysed rabbit serum. Cultures used for antigen preparation were grown in Stuart's Liquid Medium containing 8 per cent hemolyzed rabbit serum. Rabbit serum was added aseptically after medium sterilization at 15 lbs. for 15 minutes. Stuart's medium was dispensed in 90 milliliter amounts into 250 milliliter flasks. All subcultures were made with 10 per cent inoculum from 10 to 14 day-old cultures, then incubated at 30 C for 5 to 7 days. Routine microscopic examinations of a loopful of medium were performed with the darkfield microscope to determine the degree of growth. Bacteriological sterility was ascertained by plating 0.1 milliliter of each culture onto blood agar plates.

Immunization and Collection of Sera

Rabbit anti-leptospiral sera were prepared by inoculating rabbits intravenously at 5-day intervals with 1, 2, 4, 6 and 8 milliliter amounts of a suspension of leptospires. The suspensions were prepared by centrifuging 7-day old Stuart's medium cultures for 30 minutes at 3500 rcf. Sedimented cells were washed twice with phosphate buffered saline (PBS), pH 7.2. The washed cells were resuspended in PBS to one-tenth the original culture volume. Formalized cells were prepared in the same way except that after PBS washing, cells were resuspended in 0.4 per cent formalin in the same buffer. Then these cells were kept at room temperature overnight after which they were washed twice in PBS and resuspended in the same buffer to one-tenth of the original culture volume. After immunization, rabbits were bled to death. Serum was separated from the clotted blood by centrifugation and was stored at -80 C. All sera were inactivated at 56 C for 30 minutes before antibody assay.

Kinetic Studies of Antibody Synthesis During the Primary and Secondary Immune Response to L. *pomona*

Thirty-six rabbits were employed in this study. The rabbits were divided into three groups of twelve animals.

1. Twelve rabbits were inoculated intravenously with a 2 milliliter suspension of live *L. pomona* cells. This suspension had an optical density of 0.40 at a wave length of 525 millimicrons. A Spectronic 20 was used for measuring the optical density. The secondary response was elicited by giving the same dose of antigen 35 days after the primary inoculation.

2. Twelve animals were treated similarly as the above except that formalized cells were employed.

3. A third group of twelve animals served as a control.

Blood samples of approximately 4 milliliters were obtained from each rabbit. Bleedings were made from the central ear artery on days 0, 2, 3, 4, 8, 10, 14, 21, 28 and 35 after the primary inoculation and on days 37, 38, 39, 42, 46, 58 and 62 after the secondary inoculation. Serum samples were removed from clotted blood and were pooled. All sera were inactivated at 56 C for 30 minutes prior to antibody assay.

Microscopic Agglutination Test

All tests were performed in 10 mm by 75 mm test tubes. Twofold serial dilutions of the rabbit sera were made with PBS, pH 7.2 in a total volume of 0.2 milliliters. Two-tenths of a 5 to 7 day old culture of leptospires were added to each tube as the antigen. Controls containing 0.2 milliliters PBS plus 0.2 milliliters of the antigen were included in each test group. A standard antiserum was assayed with each series of tubes. After incubation in at 37 C for three hours, a drop from each tube was observed for agglutination by darkfield microscopy. The titer of the serum was determined as the highest dilution in which at least 50 per cent of the organisms were agglutinated.

Mercaptoethanol Treatment of Sera

Sera were reduced and alkylated according to the procedure of Chan and Deutsch (1960). Immune sera were mixed with an equal amount of 0.2 M 2-mercaptoethanol in PBS, pH 7.2. These mixtures were incubated at room temperature for 16 hours and were then dialyzed for 48 hours against PBS, pH 7.2. To alkylate the free sulfhydryl groups, these reduced sera were subsequently dialyzed for 48 hours against 0.02 M iodoacetamide (IA) in PBS, pH 7.2. Excess reagents were removed by dialysis for 48 hours against the same buffer. Control sera were treated in the same manner except that 2-mercaptoethanol was omitted from the dialysis buffer. Reduction of serum fractions was carried out as for the whole serum.

Heat Inactivation

One milliliter of immune sera to be inactivated was mixed with an equal volume of PBS, pH 7.2 as a protection against denaturation (Piks and Schulze, 1965). The tubes were then placed in a water bath at 65, 70, or 75 C for 30 minutes. Any precipitate which appeared during heating was removed by centrifugation before testing the clear or sometimes opalescent supernatant fluid.

Colorimetric Determination of Serum Protein

The method of DeLa Huerg et al. (1964) was employed. A standard curve was first determined by using human serum albumin (Fig. 1). One-tenth milliliter of the serum sample was mixed with two milliliters of distilled water in round 19 mm Coleman cuvettes. Four milliliters of Biuret reagent were added to each tube. The contents were allowed to stand at room temperature for at least 25 minutes. Per cent transmission was recorded at a wave length of 555 millimicrons using a Coleman Junior Spectrophotometer. The concentration of protein was determined directly from the standard curve and was expressed as grams of protein per 100 milliliters of serum.

Microzone Electrophoresis

A Beckman Model R-101 Microzone cell was employed in this study. Cellulose polyacetate membrane was used as a supporting medium. In this method, 0.25 microliters of the serum was used. Electrophoresis was carried out using barbiturate buffer, pH 8.6, of 0.075 ionic strength at 300 volts for 25 minutes. The membrane was then stained with fixative-dye (Ponceau S stain) for ten minutes, rinsed in 5 per cent glacial acetic acid, by volume in distilled water, followed by a rinse in full strength methanol. Then the cellulose polyacetate strip was cleared for one minute in a solution composed of 20 per cent glacial acetic acid and 80 per cent methanol v/v. The membrane was mounted on a glass slide and dried in a hot air oven at 100 C for 15 minutes. The percentage of serum fractions were obtained by scanning each membrane in a densitometer (Beckman Model R-110), which gave a

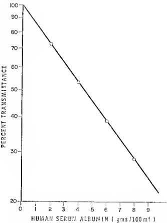

Fig. 1.—Standard curve for total protein determination.

direct paper tracing. Relative percentages of each serum fraction were then calculated from the resulting graphic figure.

Sephadex G-200 Fractionation of Sera

Serum samples of 3.5 ml, pooled from 12 rabbits, were placed on a column (2.5 cm by 95 cm) of Sephadex G-200 in which the gel was equilibrated with 0.14 M NaCl in 0.015 M Tris-HCl buffer, pH 7.4. The Sephadex G-200 was first prepared by bringing it to a boil as a suspension in distilled water to remove soluble dextran. It was equilibrated with buffer and was used to form a column. The proteins were eluted from the gel by upward flow and at a flow rate of 10 milliliters per hour. Fractions were collected in 5 milliliter volumes and protein was determined by absorbance at 280 millimicrons in a Beckman DU spectrophotometer. Selected fractions obtained from the column were subsequently tested for agglutinating activity and sensitivity to mercaptoethanol.

Ion-Exchange Chromatography

The rapid stepwise elution method of Levy and Sober (1960) was employed in this study to separate the different types of serum proteins. DEAE-cellulose, Cellex-D with a capacity of 0.70 meq per gram was used. After a series of washes in distilled water and regenerating solution (1 N NaOH, 1 N HCl), it was equilibrated in 0.015 Tris-HCl buffer, pH 8.0. The immune serum was dialyzed against the starting buffer (0.015 M Tris-HCl, pH 8.0) for 24 hours and was applied on DEAE-cellulose column (2.5 cm by 30 cm). Proteins were eluted with stepwise addition of NaCl of increasing molarity (0.05, 0.1, 0.2, 0.3 and 0.4 M) in

0.015 M Tris-HCl buffer, pH 8.0. The effluent fractions were collected in 5 milliliter volumes and were analyzed in the Beckman DU spectrophotometer at a wavelength of 280 millimicrons to obtain direct protein values. Selected fractions from each protein peak were tested for antibody activity by the microscopic agglutination test and sensitivity to 2-mercaptoethanol treatment.

Immunoelectrophoresis

The various immunoelectrophoreses were carried out on glass slides (1 by 3 inches) covered with a 2 mm layer of 1.0 per cent special Noble agar made up in 0.05 M Tris-barbital-sodium barbital, pH 8.8. Electrophoresis was carried out at room temperature in the Gelman apparatus at a constant current of 4 ma per plate (6 slides) for 60 minutes at a starting voltage of 300. Tris-barbital-sodium barbital buffer 0.05 M, pH 8.6 was used in electrode vessels and to wet the connecting wicks. Antisera were added to the trough immediately after electrophoresis. The plates were kept in a humid chamber for 48 hours at room temperature to permit development of precipitin lines. The slides were then washed, dried, and the fixed proteins were stained with Buffalo black (NBR).

Agar Double Diffusion Precipitin Tests

The method of Ouchterlony (1953) was used. A 1.5 per cent suspension of Difco-Noble agar was prepared in 0.85 per cent saline solution. After the agar was melted and cooled to about 50 C, 0.01 per cent Merthiolate was added, and a 3 ml volume was pipetted onto 25 by 75 mm glass slides. Wells of appropriate size were cut with cork borers. After the reactants were added, the slides were kept in a moist chamber for 27 to 48 hours. After precipitin lines had developed, the slides were washed, dried, and stained with Buffalo black (NBR).

Comparison of Mercaptoethanol and Heat Treatment on the 19S Activity

The sensitivity of 19S to mercaptoethanol treatment and to heat inactivation at 70 C for 30 minutes was compared. Hyperimmune serum against *L. pomona* was fractionated on Sephadex G-200. Selected fractions from the first and second protein peaks were pooled separately. Antibody titer was determined for each pool. Each pool was then divided into three aliquots and was treated as follows:

First aliquot: treated with mercaptoethanol

Second aliquot: heated in a water bath at 70 C for one hour

Third aliquot: heated in a water bath at 65 C for one hour.

Antibody activity was determined for each aliquot after the above treatments. Whole anti-leptospiral serum was treated similarly and served as a control.

Studies on the Influence of Passive Administration of Antibody on the Immunogenic Response

A. Inhibition of the primary immune response by 19S or 7S antibody. Rabbit serum hyperimmune to formalin-inactivated *L. pomona* as well as normal rabbit serum were fractionated on Sephadex G-200. Fractions comprising the first protein peak (19S) and the second protein peak (7S) were pooled separately and pressure dialyzed in the cold. These concentrated pools were used for passive administration of antibody. To determine the effect of the passive administration of 19S and

7S on the primary immune response, a group of ten rabbits were actively immunized with 2 milliliters of thoroughly washed formalized cells of L. pomona with an optical density of 0.55 at a wave length of 525 millimicrons in the Spectronic 20. Two days later, this group of animals was treated as follows:

1. Two rabbits were passively immunized with 4 milliliters of specific 7S antibodies having an agglutinin titer of 3200.

2. Two rabbits were inoculated with 4 milliliters of normal 7S globulin having an optical density in the Beckman DU at 280 millimicrons equivalent to that of the specific 7S antibodies.

3. Two rabbits were passively immunized with 4 milliliters of specific 19S antibodies having an agglutinin titer of 3200.

4. Two rabbits were inoculated with 4 milliliters of normal 19S globulin having an optical density in the Beckman DU at 280 millimicrons equivalent to that of the specific 19S antibodies.

5. The last two animals served as controls.

B. Inhibition of the primary response by passive administration of leptospiral hyperimmune serum: An experiment was designed to determine the effect of passive administration of specific rabbit anti-leptospiral hyperimmune serum on the primary response. A group of four rabbits were actively immunized with 2 milliliters of formalized L. pomona cells with an optical density of 0.55 at a wave lenth of 525 millimicrons in the Spectronic 20. Two days following active immunization the animals were treated as follows:

1. Two animals were passively immunized with 4 milliliters of rabbit anti-leptospiral serum having an agglutinin titer of 3200.

2. Two animals were injected with 4 milliliters of normal rabbit serum and served as controls.

C. Inhibition of the secondary immune response by 19S and 7S antibody: A group of the rabbits received 2 milliliters of formalized *L. pomona* cells having an optical density of 0.55 at a wave length of 525 millimicrons in the Spectronic 20. Forty-two days later all the members of this group were bled and a second equivalent dose of antigen was given. Two days following the secondary inoculation, the animals were treated as in Section A.

D. Inhibition of the secondary immune response by passive administration of leptospiral hyperimmune serum: A group of four rabbits which had received 2 milliliters of formalized *L. pomona* cells forty-two days previously were bled and a second equivalent dose of antigen was given. Two days following the secondary injection the animals were treated as in Section B.

Blood samples from each rabbit of the above four groups (A, B, C, and D) were obtained once a week following the primary and secondary injection with formalized *L. pomona* cells. Sera were separated from the clotted blood and inactivated at 56 C for 30 minutes. Microscopic agglutination tests were employed to determine the antibody titers.

RESULTS

Antibody Response to Leptospira in Rabbits

Agglutinating antibodies could be detected in the serum samples within three days after inoculation with either live cells or formalin-inactivated cells (Fig. 2). The antibody titer in the above two groups of animals arose at the same rate up to four days where the peak for formalized cells was reached. However, with live cells the antibody synthesis continued and reached a peak titer on the tenth day post inoculation.

Peak secondary microscopic agglutinin titers were reached in three days following the secondary injection. A higher titer of antibody activity was detected after a secondary injection than following a primary injection. Furthermore, it was observed that 35 days after primary injection and 47 days after secondary injection, the antibody levels in animals inoculated with live cells and with formalized cells was the same. Since a higher titer was obtained in the animals injected with live cells (Fig. 2), this indicated a sharper decline of antibody titer in the animals inoculated with live cells.

The lower agglutinin titer obtained in the case of the formalized cells might be due to the loss of specific antigenic material during formalin treatment or it may be that adequate immune response to leptospirosis, as is the situation with several other diseases of microbial origin, depends upon the presence of metabolizing organisms in the host.

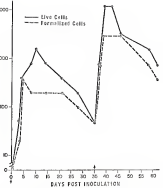

Fig. 2.—Primary and secondary agglutinin response of rabbits to live and formalin-inactivated cells of *Leptospira pomona*. Arrows indicate day of inoculation.

Mercaptoethanol Treatment of Serum Samples

Deutsch and Morton (1957) reported that macroglobulins could be dissociated by sulfhydryl reagents into smaller fragments with a molecular weight of about 160,000. A large number of investigators (Fudenberg and Kunkel, 1957; Grubb and Swahn, 1958) have provided evidence that reductive dissociation of 19S antibody resulted in the loss of its activity. In an effort to determine the characteristics of the agglutinating antibodies formed during the course of immunization with either live or formalized cells of leptospires, serum samples, taken at various intervals, were subjected to reduction with 2ME and to subsequent alkylation with iodoacetamide for the prevention of reassociation of the reduced sulfhydryl (SH) group in the immunoglobulin. Anti-leptospiral activity of early primary immune response was completely ME-sensitive up to ten days post injection (Fig. 3). On the twelfth day ME-resistant antibody appeared. Although some activity was associated with the ME-resistant antibody of sera from late primary response, the agglutinin activity was still predominantly distributed in the ME-sensitive fraction. In the secondary response (Fig. 4) the predominant fraction of antibody was also associated with the ME-sensitive fraction. This suggested that both the primary and secondary antibody responses were mainly ME-susceptible, were probably of the 19S class, and that this type of antibody might play an important role in the immunity against leptospirosis.

Sensitivity to ME of sera collected from rabbits which had been injected with formalized cells of leptospires gave a similar pattern of results (Figs. 5, 6).

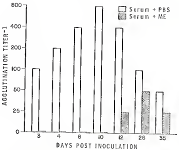

Fig. 3.—Effect of mercaptoethanol on the agglutinin titers of rabbit sera after primary inoculation with live cells of *Leptospira pomona*.

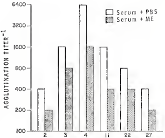

Fig. 4.—Effect of mercaptoethanol on the agglutinin titers of rabbit sera after secondary injection with live cells of *Leptospira pomona*.

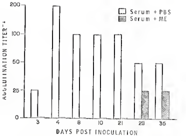

Fig. 5.—Effect of mercaptoethanol on the agglutinin titers of rabbit sera after primary inoculation with formalized cells of *Leptospira pomona*.

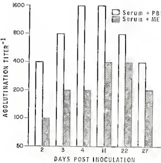

Fig. 6.—Effect of mercaptoethanol on the agglutinin titers of rabbit sera after secondary inoculation with formalized cells of *Leptospira pomona*.

Since the ME-sensitive fraction represents the level of γM antibody (evidence will be presented later) the 7S antibody titer was calculated by subtracting the ME-sensitive titer from the total activity of the serum sample. The findings are presented in Figures 7 and 8. It appeared that the sequence of production of antibodies associated with ME-sensitive and ME-resistant immunoglobulins was strikingly different during the primary response. The primary response was characterized by the initial synthesis of 19S antibody (ME-sensitive immunoglobulin) followed by the late appearance of 7S immunoglobulin which was a ME-resistant antibody. Moreover, while the ME-resistant titers were continually rising, the ME-sensitive titers were decreasing rapidly. Furthermore, the second injection of antibody was followed by prompt synthesis of a high level of ME-sensitive antibody and almost concomittant synthesis of ME-resistant antibody. The titers of both immunoglobulins during the secondary response were many times those of the primary response.

In animals inoculated with formalized cells, it was evident that the sequence of production of antibodies associated with ME-sensitive and ME-resistant immunoglobulins in the primary response was the same as described in animals inoculated with live cells (Figs. 9, 10). In the secondary response, however, the ME-resistant antibody reached a peak titer on day 56. The decline of its titer was comparatively slow in contrast to the animals inoculated with live cells where the peak secondary 7S titer was reached on the thirtyseventh day. In addition, the secondary 19S antibody reached a peak titer at day 37, which was comparable to the 19S antibody of the live cells. However,

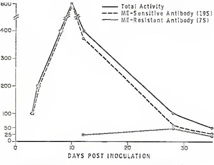

Fig. 7.—Distribution of agglutinating activity among serum samples following mercaptoethanol treatment. Rabbit sera obtained at various time intervals post primary inoculation with live cells of *Leptospira pomona*.

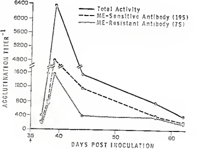

Fig. 8.—Distribution of agglutinin activity among serum samples following mercaptoethanol treatment. Rabbit sera obtained at various time intervals post secondary inoculation with live cells of Leptospira pomona. Arrow indicates time of secondary inoculation of antigen.

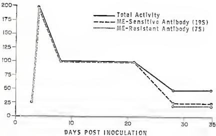

Fig. 9.—Distribution of agglutinating activity among serum samples following mercaptoethanol treatment. Rabbit sera obtained at various time intervals post primary inoculation with formalized cells of Leptospira pomona.

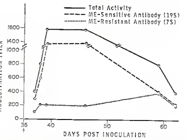

Fig. 30.—Distribution of agglutinating activity among serum samples following mercaptoethanol treatment. Rabbit sera obtained at various time intervals post secondary inoculation with formalized cells of *Leptospira pomona*. Arrow indicates time of secondary inoculation of antigen.

the 19S antibody titer was in the declining phase on day 56 when the peak 7S antibody titer was reached.

Heat Inactivation

It was apparent from the sensitivity to ME-treatment that 7M or 19S immunoglobulin was probably one of the active components produced in response to leptospiral infection. These data were by no means conclusive since a ME-sensitive 7G of mouse (Adler, 1965) and chicken immunoglobulin (Benedict *et al.*, 1963) has been reported. Locke and Segre (1965) found that inactivation at 65 C for one hour provided a more reliable measure of macroglobulin antibody than did treatment with ME. Pike and Schulze (1965) compared the difference in the heat stability of chromatographic fractions containing a predominant 7S, ME-resistant antibody and 19S, ME-sensitive antibody. Agglutinins were prepared in the rabbit against the H and O antigens of *Salmonella typhosa*, *Leptospira grippotyphosa*, bovine serum albumin, and sheep erythrocytes. Antibody in the 7S fractions were found to be more resistant to inactivation by heat than the antibody in 19S fractions. Therefore it was of interest to determine the effect of heat on agglutinating antibody titers.

Figures 11 and 12 summarize the results obtained with various serum samples. Agglutination activity of serum samples, heated at 70 C for 30 minutes, was completely destroyed up to 14 days post inoculation (Fig. 11). However, heating the same serum samples at 65 C caused no changes in the antibody titer compared to the unheated serum samples. After 21 days, antibody resistant to 70 C appeared.

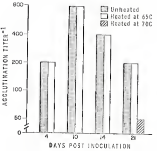

Fig. 11.—Effect of heat on the agglutinin titers of sera of rabbits after primary inoculation with live cells of Leptospira pomona.

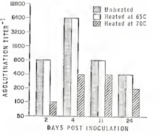

Fig. 12.—Effect of heat on the agglutinin titers of the sera of rabbits after secondary inoculation with live cells of *Leptospira pomona*.

By the fourth day following the secondary inoculation (Fig. 12), considerable activity was associated with heat-resistant antibody (70 C) fraction. In all the cases, heating serum samples at 75 C completely abolished the agglutinating antibody activity.

Comparable findings were observed when serum samples from rabbits inoculated with formalized cells were treated in the same way (Figs. 13, 14). The data presented in this experiment suggested that heat inactivation did provide information comparable to that obtained by ME-treatment.

When the titers of heat-sensitive and heat-resistant antibody were plotted against post inoculation time, it was evident that the primary response was characterized by the initial synthesis of 19S antibody, followed at day 21 by the appearance of 7S globulin antibody (Fig. 15). The secondary immune response was elicited by the increased synthesis of 19S and 7S antibody. A similar profile of antibody synthesis was observed in the animals inoculated with formalized cells (Fig. 16).

Electrophoretic Distribution of Antibodies

The data presented above showed progressive changes in the serologic properties of antibodies with time. It appeared of interest to ascertain whether those changes could be correlated with changes in the physical and chemical properties of immunoglobulins. For this purpose, pooled sera obtained at various time intervals following the first and second injections were fractionated using microzone electrophoresis. The electrophoretic patterns of the different serum samples

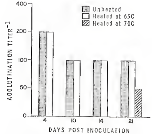

Fig. 13.—Effect of heat on the agglutinin titers of the sera of rabbits after primary inoculation with formalized cells of *Leptospira pomona*.

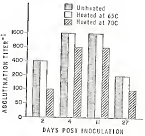

Fig. 14.—Effect of heat on the agglutinin titers of the sera of rabbits after secondary inoculation with formalized cells of *Leptospira pomona*.

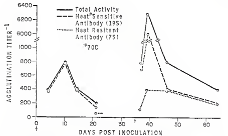

Fig. 15.—Distribution of agglutinating activity among serum samples following heat treatment. Rabbit sera obtained at various time intervals post primary and secondary inoculation with live cells of Leptospira pomona. Arrows indicate time of inoculation.

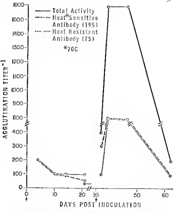

Fig. 16.—Distribution of agglutinins activity among serum samples following heat treatment. Rabbit sera obtained at various time intervals post primary and secondary inoculation with formalised cells of Leptospira pomona. Arrows indicate time of inoculation.

showed no difference. In fact, no difference in the patterns could be detected when electrophoretic patterns of normal rabbit serum were compared with the immune rabbit serum.

Quantitatively, a slight increase in the total serum protein, following inoculation or infection, was detected (Tables 1 and 2). This increase in the total serum protein was statistically significant in the serum samples obtained from rabbits which had been inoculated with live L. pomona (See Appendix).

Moreover, statistical analysis was conducted on serum fractions after densitometer measurement of the electrophoretic patterns. Results showed an increase in the α, β and γ globulins of the serum fractions (Tables 3, 4, 5).

Fractionation of Serum Samples on Sephadex G-200

In an extension of the above studies, more rigorous physico-chemical evidence was sought for the nature of the antibodies produced during the primary response and in order to gain more information about the primary and secondary antibody response. Accordingly, microscopic agglutination was employed in conjunction with fractionation on Sephadex G-200 and other methods for characterizing immunoglobulins. The two antibody responses were studied from the standpoint of the sequence of synthesis of the two molecular species of antibody.

Selected serum samples of 3.5 ml, pooled from 12 rabbits, were subjected to fractionation on Sephadex G-200. Certain fractions obtained from the column were subsequently tested for agglutinating activity and for sensitivity to ME. Evidence will be presented later

TABLE 1

TOTAL PROTEIN OF NORMAL RABBITS

Number of Rabbit	gm/100 ml
1	5.5
2	5.6
3	6.2
4	6.2
5	5.9
6	6.0
7	6.4
8	5.9
9	5.6
10	5.9
11	5.9
12	5.8

TABLE 2

TOTAL SERUM PROTEIN OF RABBITS INJECTED WITH LIVE OR FORMALIZED CELLS OF *LEPTOSPIRA POMONA*

Serum Samples*	Animals Injected With Live Cells gm/100 ml	Animals Injected With Formalized Cells gm/100 ml
1	6.7	5.1
2	6.0	5.1
3	7.3	5.8
4	6.4	5.8
5	6.5	6.4
6	6.5	6.0
7	7.1	6.0
8	7.1	6.5
9	6.6	6.3
10	6.4	6.1
11	6.9	5.9
12	6.6	6.3
13	6.7	6.5
14	6.8	6.5
15	6.9	5.9
16	6.7	6.4

*Each serum sample represents a pool of 12 rabbits.

TABLE 3

PROTEIN FRACTIONS OF NORMAL RABBIT SERUM

Number of Rabbit	gms protein/100 ml serum			
	Albumin	α	β	γ
1	4.12	0.26	0.60	0.52
2	4.26	0.32	0.59	0.43
3	4.89	0.29	0.57	0.49
4	5.01	0.30	0.55	0.34
5	4.58	0.34	0.49	0.49
6	4.74	0.25	0.50	0.50
7	4.98	0.28	0.61	0.52
8	4.78	0.19	0.58	0.36
9	4.51	0.22	0.59	0.29
10	4.79	0.23	0.55	0.32
11	4.89	0.19	0.55	0.36
12	4.58	0.34	0.49	0.40

TABLE 4

PROTEIN FRACTIONS OF IMMUNE SERA PREPARED IN RABBITS IN RESPONSE TO THE INJECTION OF LIVE LEPTOSPIRA POMONA

Serum Sample[*]	gm protein/100 ml serum			
	Albumin	α	β	γ
1	5.10	0.65	0.95	0.57
2	3.75	0.77	0.85	0.62
3	4.73	0.60	1.08	0.88
4	4.43	0.52	0.80	0.66
5	4.92	0.52	0.52	0.47
6	4.54	0.54	0.79	0.64
7	5.10	0.58	0.78	0.66
8	4.99	0.51	0.72	0.89
9	5.48	0.28	0.60	0.23
10	5.52	0.20	0.49	0.20
11	4.00	0.56	0.78	0.98
12	4.60	0.51	0.83	0.64
13	4.88	0.39	0.86	0.50
14	5.15	0.49	0.68	0.49
15	5.40	0.36	0.68	0.45
16	4.95	0.34	0.86	0.55

[*] Each serum sample represents a pool of 12 rabbits.

TABLE 5

PROTEIN FRACTIONS OF IMMUNE SERA PREPARED IN RABBITS IN RESPONSE TO THE INJECTION OF FORMALIZED *LEPTOSPIRA POMONA*

Serum Sample*	gms protein/100 ml serum			
	Albumin	α	β	γ
1	3.77	0.27	0.42	0.36
2	3.80	0.27	0.69	0.33
3	4.74	0.20	0.65	0.20
4	3.85	0.36	0.54	0.59
5	4.81	0.41	0.73	0.45
6	4.63	0.35	0.62	0.40
7	4.64	0.45	0.51	0.40
8	4.98	0.41	0.66	0.45
9	4.73	0.46	0.85	0.46
10	4.87	0.38	0.76	0.49
11	4.34	0.41	0.64	0.47
12	4.79	0.37	0.75	0.49
13	4.79	0.37	0.63	0.52
14	4.48	0.48	0.76	0.38
15	4.49	0.43	0.60	0.47
16	4.56	0.46	0.64	0.64

*Each serum sample represents a pool of 12 rabbits.

that of the three peaks obtained from Sephadex G-200, the first peak represents γM, the second peak γG, and the third peak was mainly albumin and other proteins. Therefore, in further discussion the first peak will be referred to as 19S globulin and the second peak as 7S globulin.

Typical protein elution profiles of rabbit anti-leptospiral serum are presented in Figures 17, 18, 19, 20, 21, 22, and 23. Each can be seen to consist of three discernible peaks of serum samples obtained on days 4, 8, 10, 21, 37, 29, and 62 post-inoculation. The agglutinin activity was detected only in the first peak when serum samples of 4, 8, and 10 days were fractionated. This antibody activity was completely destroyed with ME (reduction performed after fractionation). Concentration of the desired fractions of the second peak was accomplished by pressure dialysis, and these concentrated fractions were tested again for antibody activity. Although the concentration of protein in the first peak (Figs. 17, 18, 19) was 0.7, 0.68, and 0.57 milligrams per milliliter, respectively, and protein concentration in the second peak (Figs. 17, 18, 19) was 5.5, 5.0, and 6.0 milligrams per milliliter (after pressure dialysis), no antibody activity could be detected in the second peak.

Sera obtained at 21, 37, 39, and 62 days post inoculation yielded similar protein profiles but antibody activity was detected in both the first and second peaks. Antibody activity of the first peak was completely destroyed with ME. In contrast, the second peak contains ME-resistant antibody.

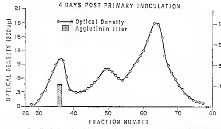

Fig. 17.—Distribution of immunoglobulins in whole rabbit anti-Leptospira pomona serum obtained by gel filtration on Sephadex G-200. Sera collected 4 days post inoculation with live cells of leptospires.

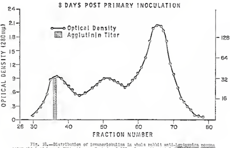

Fig. 18.—Distribution of immunoglobulins in whole rabbit anti-Leptospira pomona serum obtained by gel filtration on Sephadex G-200. Sera collected 8 days post inoculation with live cells of leptospires.

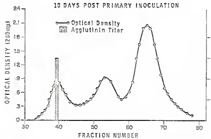

Fig. 19.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration of Sephadex G-200. Sera collected 10 days post inoculation with live cells of leptospires.

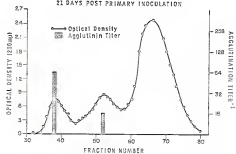

Fig. 20.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration of Sephadex G-200. Sera collected 21 days post inoculation with live cells of leptospires.

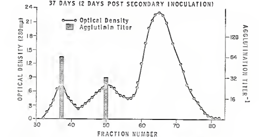

Fig. 21.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 37 days post inoculation with live cells of leptospires.

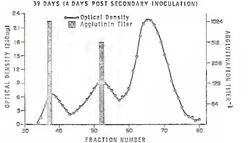

Fig. 22.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* obtained by gel filtration on Sephadex G-200. Sera collected 39 days post inoculation with live cells of leptospires.

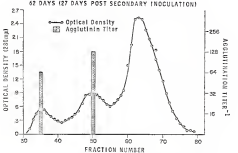

Fig. 23.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 62 days post inoculation with live cells of leptospires.

On the basis of the available data, there was a distinct difference in the sequence of molecular forms of antibody synthesized by the rabbit in response to primary injections of *L. pomona*. These data supported the previous conclusion, that the primary injection of leptospiral antigen elicited the synthesis of 19S antibody followed by the appearance of a lower molecular weight antibody species. No antibody activity could be detected in the second peak of the early fractionated sera (Figs. 17, 18, 19) and up to 21 days following the primary injection. The second injection of leptospiral cells was followed by the rapid appearance of 7S and 19S immunoglobulins with peak titers several times higher than the primary titers.

Similar observations were obtained when sera collected from rabbits after injection of formalin-inactivated cells of *L. pomona* were fractionated on Sephadex G-200 (Figs. 24, 25, 26, 27, 28, 29, 30).

Distribution of Immunoglobulins in Rabbit, Human, and Bovine Serum Fractions

It was of interest to determine the type of globulin associated with leptospiral agglutinins in the sera of rabbits, humans, and cattle. Four kinds of leptospiral antisera were used:

1. Antiserum was prepared in rabbits against several injections of increasing doses of *L. pomona* (pathogenic serotype).

2. Antiserum was prepared in rabbits against several injections of increasing doses of *L. biflexa* (a saprophytic serotype).

3. Antiserum was obtained from humans naturally infected with *L. icterohaemorrhagiae*.

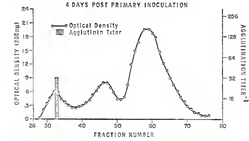

Fig. 24.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 4 days post inoculation with formalized cells of leptospires.

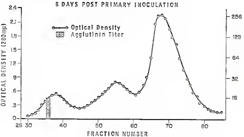

Fig. 25.—Distribution of immunoglobulins in whole rabbit anti-Leptospira pomona serum obtained by gel filtration on Sephadex G-200. Sera collected 8 days post inoculation with formalized cells of leptospires.

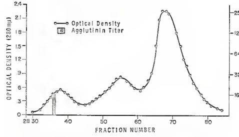
10 DAYS POST PRIMARY INOCULATION

Fig. 26.—Distribution of immunoglobulins in whole rabbit anti-Leptospira pomona serum obtained by gel filtration on Sephadex G-200. Sera collected 10 days post inoculation with formalized cells of leptospires.

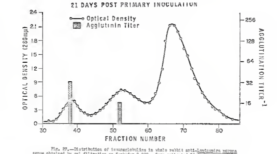

Fig. 27.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 21 days post inoculation with formalized cells of leptospires.

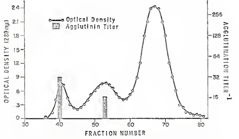
37 DAYS (2 DAYS POST SECONDARY INOCULATION)

Fig. 28.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 37 days post inoculation with formalized cells of leptospires.

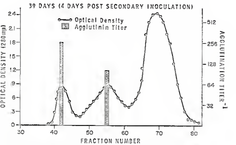

Fig. 29.—Distribution of immunoglobulins in whole rabbit anti-Leptospira pomona serum obtained by gel filtration on Sephadex G-200. Sera collected 39 days post inoculation with formalized cells of leptospires.

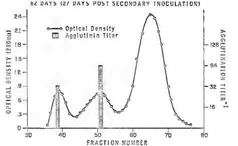

Fig. 30.—Distribution of immunoglobulins in whole rabbit anti-*Leptospira pomona* serum obtained by gel filtration on Sephadex G-200. Sera collected 62 days post inoculation with formalized cells of leptospires.

4. Antiserum was prepared in bovines against a single, large dose of *L. pomona*.

Two major components were obtained when immune sera were subjected to Sephadex G-200 gel filtration (Figs. 31, 32, 33, 34). The excluded peak (first peak) contained, as expected from similar gel filtration experiments with early sera, the ME-sensitive antibody. The second peak contained the ME-resistant antibody activity present in the applied samples. In the case of immune sera prepared against *L. pomona* (whether the agglutinins were prepared in rabbits or cattle), it was of interest to notice that the agglutinin activity of the first peak was completely destroyed when treated with 2ME (Figs. 35, 36). However, the agglutinin activity of the excluded peak (19S) of serum prepared in rabbits against *L. biflexa* was markedly reduced but not completely destroyed by ME treatment (Fig. 37). Immunoelectrophoresis studies excluded the possibility that the first peak (19S) ME-sensitive was contaminated with 7S antibody, ME-resistant.

Similar results to those obtained with rabbit and cattle anti-*L. pomona* were obtained with human anti-*L. icterohemorrhagiae* (Fig. 38).

Further characterization of anti-*L. pomona* hyperimmune serum was attempted. Each of the major peaks collected by gel filtration was examined for homogeneity by immunoelectrophoresis and Ouchterlony techniques using goat anti-whole rabbit serum.

Selected fractions were concentrated to about one-tenth their original volume, yielding preparations containing 2.5 to 3.5 milligrams of protein per milliliter. Protein concentrations were estimated from absorbance at 280 millimicrons assuming $E_{20\ C}^{1\%}$ = 1.4 milligrams of protein per milliliter.

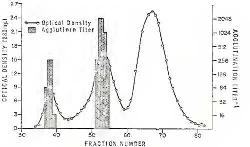

Fig. 31.—Distribution of agglutinins in fractions of rabbit anti-Leptospira pomona serum eluted from Sephadex G-200.

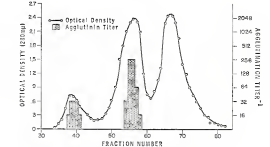

Fig. 32.—Distribution of agglutinins in fractions of bovine anti-Leptospira pomona serum eluted from Sephadex G-200.

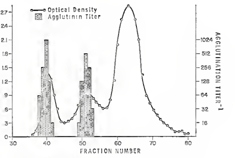

Fig. 33.—Distribution of agglutinins in fractions of rabbit anti-*Leptospira biflexa* serum eluted from Sephadex G-200.

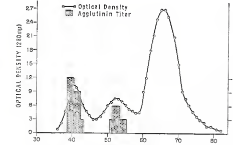

Fig. 34.—Distribution of agglutinins in fractions of human anti-Leptospira icterohemorrhagiae serum eluted from Sephadex G-200.

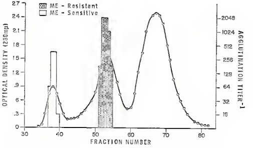

Fig. 35.—Effect of mercaptoethanol on the agglutinin titers of the separated fractions of rabbit anti-*Leptospira pomona*.

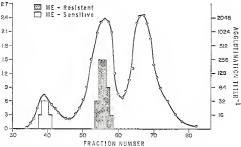

Fig. 36.—Effect of mercaptoethanol on the agglutinin titers of the separated fractions of bovine anti-*Leptospira pomona*.

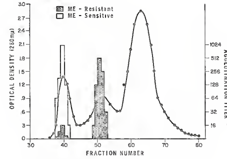

Fig. 37.—Effect of mercaptoethanol on the agglutinin titers of the separated fractions of rabbit anti-*Leptospira biflexa*.

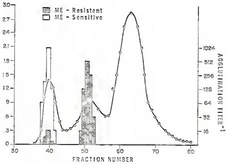

Fig. 37.—Effect of mercaptoethanol on the agglutinin titers of the separated fractions of rabbit anti-*Leptospira biflexa*.

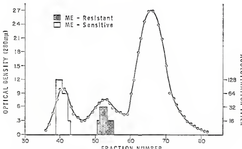

Fig. 38.—Effect of mercaptoethanol on the agglutinin titers of the separated fractions of human anti-*Leptospira icterohemorrhagiae*.

Each of the two peaks contained multiple antigens. The first peak (19S) (Fig. 39) contained two distinct components migrating in γ_2M and γ_1 (IgM) regions and no γ_2 globulins (7S globulin). The second peak (Fig. 40) contained mainly γ_2 globulin (presumably IgG gamma globulin) and some contamination with albumin but no detectable components of the first peak. In addition, the two protein fractions were antigenically analyzed in Ouchterlony slides to reveal any antigenic relationship between them. Figure 41 presents an Ouchterlony slide where it can be seen that the two protein fractions were antigenically related and formed partial identity. Moreover, selected pooled and concentrated fractions of the first protein peak were treated with 2ME and then immunoelectrophoresis was carried out on this preparation. No precipitin arcs could be detected. This was probably due to the fact that 2ME splits the γ_2 and γ_1 macroglobulins into their subunits, and these subunits lack the capability of producing a precipitin reaction in the gel.

Purification of Immunoglobulins

In an initial attempt to purify immunoglobulins for physico-chemical studies, rabbit anti-L. pomona hyperimmune serum (prepared in response to live cells) was subjected to gel filtration on Sephadex G-200. These column profiles showed a high amount of both macroglobulin (19S) and smaller molecules (7S) gamma globulin. Although this procedure readily separated the two molecular species of rabbit antibody, Ouchterlony analysis, using goat anti-sera to rabbit serum, indicated that each antibody-containing fraction consisted of multiple antigens.

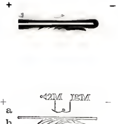

Fig. 39.—Immunoelectrophoretic analysis of fractions of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the first protein peak from gel filtration.

b. The lower well is whole rabbit serum.

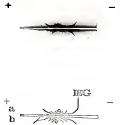

Fig. 40.—Immunoelectrophoretic analysis of fraction of rabbit anti-Leptospira pomona serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the second protein peak from gel filtration.

b. The lower well is whole rabbit serum.

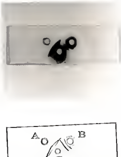

Fig. 41.—Ouchterlony analysis of rabbit anti-leptospiral immunoglobulins. IgG and IgM are separated by gel filtration. A contains IgG, B contains IgM, and C contains goat anti-rabbit whole serum.

Furthermore, immunoelectrophoresis studies indicated considerable mobility differences between the proteins within each fraction. Therefore, ion-exchange chromatography was employed as an initial purification step.

Fractionation on Diethylaminoethyl-Cellulose (DEAE-Cellulose)

Anti-leptospiral hyperimmune serum was dialyzed for 18 hours against 0.015 M Tris-HCl buffer, pH 8.0. After dialysis it was applied to a DEAE-cellulose column. Protein was eluted with stepwise addition of increasing molarity of NaCl (0.05, 0.1, 0.2, 0.3, and 0.4 M) in 0.015 M Tris-HCl buffer. Selected fractions from each peak were tested for antibody activity and sensitivity to ME. Figure 42 shows the distribution of agglutinins of rabbit anti-leptospiral serum eluted from DEAE-cellulose. Six protein peaks were demonstrated. The agglutinin activity was found to be distributed among the first five protein peaks. No antibody activity could be detected in the sixth peak. The effect of ME on the agglutinating activity of all the peaks was determined. Only the agglutinating activity of the fifth peak was completely destroyed (Fig. 43). This suggested that the first four protein peaks contained antibody of molecular species resistant to ME and was probably IgG. The complete destruction of the activity of the fractions of the fifth protein peak demonstrated that it contained ME-sensitive antibody, probably IgM. To confirm the above assumption, selected fractions from each protein peak were pooled separately and were pressure dialyzed in the cold to give a protein concentration of 2.5 to 3.0 milligrams per milliliter. These fractions were then

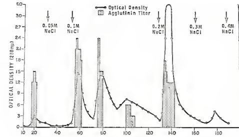

Fig. 42.—Ion exchange chromatography on DEAE-cellulose of rabbit anti-*Leptospira pomona* hyperimmune serum showing agglutination activity.

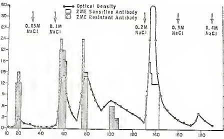

Fig. 43.—Ion exchange chromatography on DEAE-cellulose of rabbit anti-*Leptospira pomona* hyperimmune serum showing agglutination activity following mercaptoethanol treatment.

analyzed by immunoelectrophoresis. Figures 44, 45, 46, 47, and 48 show the immunoelectrophoretic patterns of the above concentrated fractions. It was clear that the first four protein peaks contained IgG. The second protein peak was composed of pure IgG (Fig. 45). The first, third, and fourth protein peaks showed a contamination with more negatively charged protein, but no detectable components in the γ_1-region (19S globulin or IgM). Immunoelectrophoretic patterns of the fifth peak showed that this peak was composed of protein components migrating in the γ_1 regions and a contamination with more negatively charged proteins (Fig. 48).

Further purification of antibody activity of the fifth peak was attempted. Selected fractions were pressure dialyzed and then were equilibrated in 0.14 M NaCl in 0.015 M Tris-HCl buffer, pH 7.4. The dialyzed fraction was applied onto Sephadex G-200. Fractions of 5 ml were collected. Protein fractions which were excluded (first peak) from the column were tested for antibody activity and for sensitivity to ME. Immunoelectrophoretic analysis suggested that the excluded protein peak contained a single protein migrating in the γ_1 (IgM or 19S) region (Fig. 49).

Furthermore, antigenic analyses were conducted to ascertain the purity of the anti-leptospiral immunoglobulins (IgM and IgG) as well as to reveal any antigenic relationship between them. Figure 50 presents an Ouchterlony slide which showed a relative antigenic purity of the two antibody species as well as a reaction of partial identity. A reaction of complete identity is shown in Figures 51 and 52.

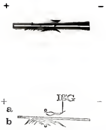

Fig. 46.—Immunoelectrophoretic analysis of a fraction of rabbit anti-_Leptospira pomona_ serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the first protein peak from diethylaminoethyl (DEAE) chromatography.

b. The lower well is whole rabbit serum.

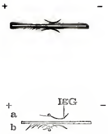

Fig. 45.—Immunoelectrophoretic analysis of fraction of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the second protein peak from diethylaminoethyl (DEAE) chromatography.

b. The lower well is whole rabbit serum.

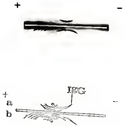

Fig. 46.—Immunoelectrophoretic analysis of fraction of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. In the upper well is the third protein peak from diethylaminoethyl (DEAE) chromatography.

b. The lower well is whole rabbit serum.

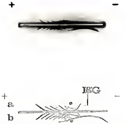

Fig. 47.—Immunoelectrophoretic analysis of fraction of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the fourth protein peak from diethylaminoethyl (DEAE) chromatography.

b. The lower well is whole rabbit serum.

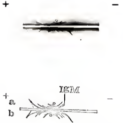

Fig. 48.—Immunoelectrophoretic analysis of a fraction of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. The upper well is the fifth protein peak from diethylaminoethyl (DEAE) chromatography.

b. The lower well is whole rabbit serum.

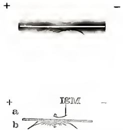

Fig. 49.—Immunoelectrophoretic analysis of fraction of rabbit anti-*Leptospira pomona* serum. Goat anti-rabbit serum is in the trough.

a. The upper well is IgM globulin after diethylaminoethyl (DEAE) chromatography and gel filtration.

b. The lower well is whole rabbit serum.

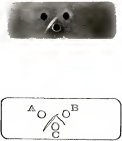

Fig. 50.—Ouchterlony analysis of rabbit anti-leptospiral immunoglobulins. A contains IgG, B contains IgM, and C contains goat anti-rabbit whole serum.

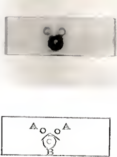

Fig. 51.—Ouchterlony analysis of rabbit anti-leptospiral IgM. A contains IgM eluted from diethylaminoethyl (DEAE) chromatography and gel filtration. B contains goat anti-rabbit whole serum.

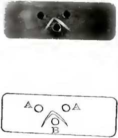

Fig. 52.—Ouchterlony analysis of rabbit anti-leptospiral IgG. A contains IgG eluted from diethylaminoethyl (DEAE) chromatography. B contains goat anti-rabbit whole serum.

Comparison of Mercaptoethanol and Heat Treatment on the 19S Activity

In an effort to compare the sensitivity of the 19S globulin to ME-treatment and to heat inactivation (70 C), hyperimmune serum against *L. pomona* was fractionated on Sephadex G-200. Selected fractions from the first and second protein peaks were pooled separately and were treated as described under the materials and methods. Table 6 summarizes the results. It was obvious that ME was more sensitive in detecting 19S immunoglobulin. Complete destruction of activity was observed when fractions of the first protein peak (19S) were treated with ME. However, 19S antibody activity was partially destroyed at 70 C. When whole serum was treated similarly, it was of interest to note that ME and heat treatment had the same effect on antibody activity. This suggested that other serum proteins probably had some protective effect on 19S immunoglobulin.

As expected, 7S antibody against *L. pomona* withstood a temperature of 70 C for one hour as well as treatment with ME.

The 19S Antibody Response to *L. pomona*

Several studies concerned with the synthesis of high molecular weight (γM or 19S) and low molecular weight (γG or 7S) antibodies in rabbits and guinea pigs have led to the conclusion that although 19S antibody is usually the first to appear in the serum after initial injection, the 19S response, nevertheless, is only short-lived. However, in the present study 19S (γM) antibody was observed for long periods of time in both primary and secondary responses. In fact, the

TABLE 6

EFFECT OF 2-MERCAPTOETHANOL AND HEAT ON γM IMMUNOGLOBULIN

Treatment	Antibody Titer 1		
	Whole Serum	γM	γG
No treatment	12,800	256	1024
Heating at 65 C for 1 hour	12,800	256	1024
Heating at 70 C for 1 hour	6,400	16	1024
2-mercaptoethanol	6,400	-	1024

primary response consisted mainly of 19S (γM) antibody and considerable activity could be detected in the first protein peak when late serum samples were fractionated (i.e. on day 62 post inoculation). In an effort to gain more information on the persistance of 19S antibody response to the L. pomona antigen, a serum sample obtained six months post inoculation was fractionated on Sephadex G-200. From Figures 53 and 54 it was evident that although the majority of antibody activity was associated with 7S (the second peak), some activity was still associated with the 19S antibody (first peak). The results were contradictory to most of the reports of other bacterial infections in rabbits (Bauer and Stavitsky, 1961; Bauer et al., 1963; Svehag and Mandel, 1962; Uhr and Finkelstein, 1963) since 19S antibody appeared to be synthesized for a long period of time after inoculation (i.e. 180 days).

Studies on the Influence of Passive Administration Antibody on the Immunogenic Response

The immunosuppressive power of rabbit anti-leptospiral whole serum was tested in rabbits actively immunized with formalized L. pomona. A significant reduction in the agglutinin titer was observed as a result of passive administration of whole anti-leptospiral immune serum (Fig. 55). Passive administration of whole normal rabbit serum had no immunosuppressive effect.

In addition, inhibitory power of specific 7S and 19S immunoglobulins against L. pomona, after separation using Sephadex G-200, was observed. Specific 7S antibody injected two days following active immunization suppressed the immunogenic response (Fig. 56). Normal rabbit 7S globulin had no effect on the response to antigenic stimulation.

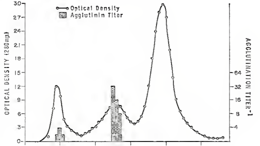

Fig. 53.—Distribution of agglutinins in fractions of rabbit serum eluted from Sephadex G-200 six months after intravenous injection of live cells of *Leptospira pomona*.

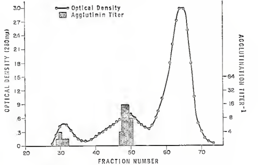

Fig. 54.—Distribution of agglutinins in fractions of rabbit serum eluted from Sephadex G-200 six months after intravenous injection of formalized cells of *Leptospira pomona*.

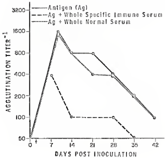

Fig. 55.—Suppression of antibody formation to Leptospira pomona in rabbit by passive administration of rabbit anti-leptospiral whole serum. Arrow indicates time of passive administration.

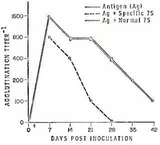

Fig. 56.--Suppression of antibody formation to *Leptospira pomona* in rabbit by passive administration of rabbit anti-leptospiral 7S immunoglobulin. Arrow indicates time of passive administration.

Figure 57 shows the results of a similar experiment using 19S immunoglobulin in an amount equivalent in serological activity (as measured by the microscopic agglutination test) to that of the 7S immunoglobulin used in the above experiment. Neither specific 19S antibody nor normal 19S globulin had a significant immunosuppressive effect in the amounts used.

Experiments were also designed to determine the effect of passive intravenous administration of whole immune sera and the effect of 19S and 7S immunoglobulins on the secondary response to the antigen. In contrast to the primary response, no immune suppression could be detected as a result of passive administration of antibodies followed by secondary active immunization with leptospires (Figs. 58, 59, 60).

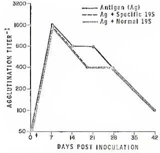

Fig. 57.—Effect of passive administration of rabbit anti-leptospiral 19S immunoglobulin on the response to active immunization with leptospires. Arrow indicates time of passive administration.

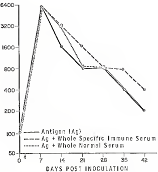

Fig. 58.—Effect of passive administration of rabbit whole anti-*Leptospira pomona* serum on the secondary response to leptospiral antigenic stimulation. Arrow indicates time of passive administration.

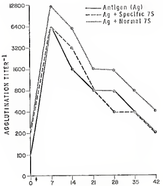

Fig. 59.—Effect of passive administration of rabbit antileptospiral 7S immunoglobulin on the secondary response to Leptospira pomona antigenic stimulation. Arrow indicates time of passive administration.

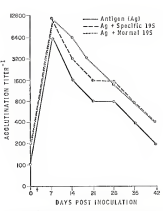

Fig. 60.—Effect of passive administration of rabbit anti-leptospiral 19S immunoglobulin on the secondary response to *Leptospiral pomona* antigenic stimulation. Arrow indicates time of passive administration.

DISCUSSION

Nature of Antibody Response in Rabbits Inoculated with *L. pomona*

Physicochemical evidence was presented in this study for the synthesis of two distinct species of antibody during the primary and secondary responses of rabbits to intravenous injection of *L. pomona*. Following the injection of any one of various antigens into humans (Lo Spalluto *et al.*, 1962; Fink *et al.*, 1962; Uhr *et al.*, 1962a) and rabbits (Stelos and Taliaferro, 1959; Bauer and Stavitsky, 1961; Benedict *et al.*, 1962), rapidly sedimenting antibody (19S) first appears in the circulation, and later is replaced by molecules of 7S sedimentation constant. Similar results were obtained after injection of bacteriophage φX 174 into guinea pigs (Uhr *et al*, 1962b), and several non-mammalian vertebrates (Uhr *et al.*, 1962c), indicating the general occurrence of this sequence in the immune response.

In the present study, results indicate similar molecular heterogeneity of antibody produced by a single injection of antigen. This heterogeneity was demonstrated by sensitivity to sulfhydryl reagent, by sensitivity to heating at 70 C for 30 minutes, and by gel filtration of the antisera into different fractions and subsequent assay of these fractions for antibody by microscopic agglutination tests. Evidence revealed that two types of antibody were synthesized as a result of antigenic stimulation. There was distinct evidence for sequential synthesis of antibody by rabbits in response to primary injection of live and formalin-inactivated *L. pomona*. The data particularly supported the conclusion that the primary injection of antigen elicited the synthesis of γM antibody followed by the appearance of γG antibody. The early sera contained only γM as shown by ME-inactivation, heat inactivation, and Sephadex fractionation of sera. The activity associated with the later response (21 to 35 days after primary injection) involved the presence of γG as well as of γM. The secondary stimulation with *L. pomona* was followed by rapid appearance of γG globulin with a higher peak titer and a concomitant synthesis of γM antibody. This second injection of antigen apparently triggers immediate 7S immunoglobulin synthesis contrary to the primary response in which case 7S synthesis was preceded by the appearance of 19S antibodies.

Several investigators, using such methods as the Farr antigen-binding technique and radioimmunoelectrophoresis, have reported the appearance of early 7S antibody activity in various systems (Benedict, 1965; Freeman and Stavitsky, 1965; Cohen *et al.*, 1967; Cushing and Johnson, 1966). These reports contradicted the concept of sequential synthesis of immunoglobulins.

In the present study, γM was found to be the only active component in the early immune response to *L. pomona*, and it was the predominant class of immunoglobulin throughout the period of immunization. However, it must be stressed that the detection of one or the other immunoglobulin is greatly dependent upon the type of assay employed (Grey, 1964; Benedict, 1965; Robbins *et al.*, 1965; Cushing and Johnson, 1966; Cohen *et al.*, 1967). Therefore, the fact that γG was not detected earlier in the immune response might simply be a reflection of the greater sensitivity of γM in the microscopic-agglutination test.

It was of interest to observe that injection of rabbits with leptospires induced an increase in the various globulin fractions (α, β, and γ). The possibility that the increase in γ-globulin can be considered as a manifestation of the immune response is complicated by the problem of natural variations.

The most provocative finding to emerge from the present study is the fact that γM immunoglobulin was synthesized throughout the period during which the immune response was studied (Figs. 53, 54). This observation is contrary, therefore, to most of the previous reports in which γM synthesis ceased shortly after immunization. Moreover, there was a rapid rise in γM titer immediately following the secondary inoculation. These findings are in agreement with previous reports by Nocker and Bauer (1965) and Nossal *et al.* (1964) who demonstrated the rapid appearance of cells producing 19S anti-flagellar antibodies after reinjection with *Salmonella*. This continued synthesis of macroglobulin antibody could be a result of the persistance of the particular antigen which evokes the γM response (Uhr and Finkelstein, 1963; Svehag and Mandel, 1964).

The possibility that the observed increased synthesis of γM following reinjection with *L. pomona* might be considered to be a real anamnestic response was complicated by the fact that quantitative estimation of γM and γG immunoglobulins were not carried out.

Preliminary characterization of the two kinds of antibody (γM and γG) of the hyperimmune serum was attempted. Rabbit γM and γG immunoglobulins against L. pomona were isolated from two fractions of immune sera prepared by Sephadex G-200 and DEAE-cellulose chromatography. The two protein preparations were examined by several immunochemical criteria, with results which justified their identification as γM and γG globulins.

The identity of immune specificity was based upon specific bacterial agglutination of L. pomona. However, the two kinds of immunoglobulins differed markedly in their physicochemical properties. Treatment of the γM antibody with 2ME resulted probably in the dissociation of the intact molecules into subunits which completely lost their serological activity. The loss of agglutination activity of the macroglobulin fractions after treatment with ME is probably due to reductive cleavage of the macroglobulin. This loss of activity of γM immunoglobulin upon treatment with ME was in direct contrast to failure to affect the serological activity of γG antibody. Moreover, the γM fraction contained a protein which showed fast gamma mobility by immunoelectrophoresis. In contrast, the γG fraction formed a single line of precipitin in the slow gamma region.

Heating of the γM immunoglobulin at 70 C for 30 minutes resulted in considerable reduction in agglutinin activity, whereas γG immunoglobulin resisted heating at 70 C for 30 minutes. In addition, γM globulin was found to be more sensitive to ME treatment than to heat inactivation (at 70 C). This finding indicated that ME inactivation is a more reliable criterion to distinguish immunoglobulin activity in serum fractions than is inactivation by heat.

Studies on the Influence of Passive Administration of Antibody on the Immunogenic Response

The study reported here suggests that the capacity of passively administered antibody to suppress antibody formation was mainly dependent upon the type of immunoglobulin used. The 7S immunoglobulin was shown to be capable of suppressing the formation of antibody to a significant degree (Fig. 56). This finding is in agreement with previous reports (Finkelstein and Uhr, 1964; Möller and Wigzell, 1965). However, a number of investigations have indicated that both 19S and 7S immunoglobulins were capable of suppressing the formation of antibody (Pearlman, 1967; Wigzell, 1966; Rowley and Fitch, 1964). Differences in these results may relate partly to differences in methods used to assay the activity of 7S and 19S immunoglobulins administered. In the present study Sephadex G-200 was employed for the separation of serum fractions. The amounts of 19S and 7S antibodies used in passive immunization were based solely on the serological activity (microscopic agglutination). The fractions were adjusted so that they were comparable with regard to agglutinin titers. The results demonstrated that 7S antibodies were more efficient than 19S antibodies in causing immune suppression. A possible explanation is that 19S antibody is more efficient on a molecular basis in immune agglutination, and that, therefore, the amount of 19S immunoglobulin administered, although representing the identical titer as that of the 7S antibody, may be below the critical level of antibody required to cause immunosuppression.

Pearlman (1967) reported that the ability to suppress antibody formation was not a property exclusive to a particular immunoglobulin type. The 19S and 7S antibodies were both shown to be capable of suppressing the formation of antibody to significant degrees. The amounts of 19S and 7S antibodies used for passive administration were based on relative combining capacity. The author suggested that the apparent discrepancy between this report and other reports probably reflected the fact that suppression of antibody formation is more a function of combining activity than of other serological properties of antibody molecules. However, the author has not shown that there is any difference in the combining capacity of the 19S and 7S antibodies. In addition, the faster catabolic rate of 19S compared with that of 7S antibody may be responsible for the difference in effectiveness observed (Taliaferro and Talmage, 1956). Different catabolic rates have also been implicated as a possible cause of the reduced effectiveness of pepsin-digested antibody compared with the same amount of undigested antibody in suppressing antibody formation (Tao and Uhr, 1966).

Since it has been suggested that avidity is one of the decisive factors in the suppression of antibody on antibody formation, critical differences between the 19S and 7S preparations may well have existed in this respect.

Although the mechanism by which antibody exerts its inhibitory effects is not clear, it has been postulated (Uhr and Baumann, 1961a; Finkelstein and Uhr, 1964) that the suppressive effect of passive antibody is the result of its ability to bind antigen and thus to prevent it from stimulating active antibody synthesis. An alternative mechanism of inhibited response may be explained by the hypothesis of Brody et al. (1967). According to this hypothesis, the stimulation of immunocompetent cells to proliferate and produce antibody may be dependent upon the interaction of antigen with pre-formed antibody present on or in these cells. Such a "selection" theory presumes that the cells precommitted to respond to a particular antigen are present in the animal prior to contact with antigen. Furthermore, it was hypothesized that this cell-associated antibody has a binding site identical with that of the antibody molecules to be produced by the cell after interaction with antigen. Passively administered antibody competes with cell-associated "antibody" for available antigen molecules. Once bound to circulating antibody, the antigenic determinant is no longer available for binding cell-associated antibody and thus is incapable of triggering cell proliferation and antibody synthesis.

Neither hyperimmune whole serum nor 19S and 7S immunoglobulins exerted any immunosuppressive effect on the secondary response (Figs. 58, 59, 60). The failure of passively administered specific antibody to produce a significant immunosuppression during the secondary response is in general agreement with previous reports (Neiders et al., 1962; Rowley and Fitch, 1964). It seems likely that the secondary response represents the net effect of inhibition of the response by antibody and enhanced reactivity of the host produced by stimulation of previous specific immunization. A possible explanation is that, during a secondary response, the antibody-forming cells can successfully compete for available immunogen with the passively administered antibody. However, there is no experimental evidence to support this contention.

The inhibition of antibody response in the passively immunized animal may be comparable to immunological enhancement of tumor homografts (Kaliss, 1958; Snell, 1957), where it was necessary for passive antibody to be present in order to suppress the immunological response to antigen (Wallis and Melnick, 1962). Therefore, it seems likely that inhibition of antibody synthesis is due to the non-availability of antigen or "processed antigen" which might be essential to elicit antibody synthesis.

Dean and Webb (1928) observed that the dose of antigen required to elicit the secondary response is much less than that required to cause a primary response. In addition, Ada et al. (1968) have reported that during the primary response the antigen is extensively distributed throughout the medulla and lymphoid follicles of the regional lymph nodes. In contrast, a secondary antibody response could be fired by antigen without its being extensively trapped in either the lymph node medullary macrophages or lymphoid follicles. Thus, it seems that much less antigen is needed to trigger off the secondary response. This would also explain why large amounts of antibodies are required to suppress the secondary response. In the present study, the amount of passively administered antibody seems to be sufficient to prevent extensive trapping of antigen, thus, creating the condition of "non-availability of antigen" subsequently suppressing the primary response. However, this amount of antibody might allow at least enough antigen-trapping to evoke the secondary immune response.

All of these findings suggest that the ability of antibodies to suppress their own synthesis constitutes a primary homeostatic mechanism. Not only passively transferred hyperimmune sera, but also endogenously produced antibodies in the primary response are competent to suppress antibody synthesis.

SUMMARY

The primary and secondary immune responses in rabbits following intravenous injection of *L. pomona* were studied. Rabbit anti-*L. pomona* antibodies were isolated by combined physical and immuno-specific methods, and some of their properties in the immunological system were determined. Moreover, the effect of massive administration of leptospiral antibodies on the immunogenic response to *L. pomona* was investigated. The following points were demonstrated.

1. After primary inoculation with either live or formalin-killed *L. pomona*, the rabbits responded with a rapid production of agglutinating antibody. Inactivation of sera with heat, ME and Sephadex fractionation indicated that the observed agglutinating activity was associated with a macroglobulin which was completely ME-sensitive. The activity associated with the later response (21 days) involved the presence of γG as well as γM immunoglobulin.

2. The secondary response to the same antigen was comprised of the early synthesis of large amounts of both kinds of antibodies, γM and γG.

3. The unusual feature of the immunological response, the persistence of γM for long periods of time (at least six months), was demonstrated.

4. Rabbit γM and γG antibodies against *L. pomona* were isolated from two fractions of immune sera prepared by DEAE-cellulose chromatography and Sephadex gel filtration. The two protein preparations

were examined by several immunochemical criteria, with results which indicated their identification as γM and γG antibodies.

5. Passive inoculation of rabbits with anti-*L. pomona* serum significantly inhibited the primary response to antigenic stimulation by leptospires.

6. The immunosuppression was specific and apparently was produced by 7S immunoglobulin.

7. The immunosuppressive effect of specific antibody on the primary response contrasted sharply with the absence of this effect on the secondary response.

APPENDICES

APPENDIX A

SOME TECHNIQUES USED IN THIS STUDY

Biuret Reaction

This is a colorimetric method of protein analysis. This reaction depends essentially upon the formation of a complex in alkaline solution in which one atom of copper is bound to four peptide nitrogens (Mehl *et al.*, 1949).

Fractionation on Sephadex G-200

The sieve properties of a particular gel are a function of the porosity of the gel particle which is determined by the degree of cross-linkage between dextran chains. Sephadex G-200 has the largest pore size. This allows the separation of equivalent Stokes radii. For a given gel, particle size separation is a direct function of the number of "theoretical plates" (Flodin and Killander, 1962). The number of the theoretical plates, N, is given by the formula $N = L/EHTP$, where L is the column length and EHT is the equivalent height of a theoretical plate, which, for a given particle size, can be decreased by reducing the flow rate.

Because of the great compressibility of the large pore-sized Sephadex G-200 particles, increasing the bed height can result in flow rates so slow as to be impractical. The technique of reverse flow (solvent flow up the column) was used in the present study in order to minimize or avoid the compaction of the gel material (Rothstein, 1965; Porath and Bennich, 1962).

Immunodiffusion Technique

This is a technique developed by Oudin (1946). With this method the antigen-antibody precipitation takes place in a gel medium. Due to the different diffusion rates of the heterogeneous antigens, the differentiation of various antigens is made possible. The double diffusion technique, i.e., diffusion of antigens and antibodies from opposite directions, results in the formation of multilayered precipitations if heterogeneous antigen solutions and polyvalent antisera are used.

Electrophoresis

Electrophoresis is the movement of particles or ions by direct current through a conducting solvent (electrolyte), using buffered water. The basic apparatus consists of a power supply for direct current, positive and negative non-polarizing electrodes, a vessel of buffer for immersing each electrode, and some kind of bridge connecting these two buffer vessels and acting as the support for the material in which the electrophoresis will occur.

Immunoelectrophoresis

These are electrophoretic and agar diffusion methods for the resolution and identification of the antigens present in complex mixtures.

APPENDIX B

STATISTICAL ANALYSES

Statistical Model: One Way Analysis of Variance

$y_{ij} = \mu + \alpha_i + \epsilon_{ij}$ $\qquad i = 1, 2, 3$

$\qquad\qquad\qquad\qquad j = 1, 2, \ldots j_i$

Where:

y_{ij} = response of rabbits to inoculation i

μ = mean response for all rabbits

α_i = affect of inoculation i

ϵ_{ij} = error; a random variable distributed normal with mean zero and variance σ^2 (unknown)

All errors are assumed to be independent.

Hypothesis: Inoculation has negligible effects ($\alpha_i = 0$).

Test at 0.05 level of significance.

Pr—rejecting hypothesis given that it is in fact true = 0.05

Analysis of Variance Table (N = 44)

Source	S.S.	D.F.	M.S.
Between groups	$\Sigma_i\, j_i(y_i - \bar{y})^2$	2	SS/2
Within groups	$\Sigma_i\, \Sigma_j(y_{ij} - y_i)^2$	41	SS/41
Total	$\Sigma_i\, \Sigma_j(y_{ij} - \bar{y}_i)^2$	43	

Sheffé Method of Multiple Comparison

Conclude that pairwise effects are significantly different if:

$$|y_i - y_i'| > S\hat{\sigma} \quad \text{where} \quad S = [(I-1)F_{\alpha,\, I-1,\, n-I}]^{\frac{1}{2}}$$

$$\hat{\sigma} = [MSE(1/J_i + 1/J_i)]^{\frac{1}{2}}$$

$$MSE = SS/n - I$$

For all data: $\alpha = 0.05$

$$S = [2(3.23)]^{\frac{1}{2}} = 2.54 \quad \text{Standard deviation}$$

for case (a) $J_i = J_i' = 16 \sqrt{1/J_i + 1/J'} = 0.354$

$$S\hat{\sigma} = 0.9 \sqrt{MSE}$$

case (b) $J_i = 12$ $J_i' = 16 \sqrt{1/J_i + 1/J'} = 0.382$

$$S\hat{\sigma} = 0.97 \sqrt{MSE}$$

Statistical Analysis of Total Serum Protein in Rabbits Injected with Live or Formalized Cells of *L. pomona*

$\sum_{ij} y_{ij} = 274.1$ $\qquad$ $\sum_{i}\sum_{j} y_{ij}^2 = 1717.73$

$\bar{y} = 6.2295$ $\qquad$ $\bar{y}^2 = 38.8067$

i	y_i	y_i^2	J_i	s_i^2
1	5.9083	34.9080	12	0.1092
2	6.0375	36.4514	16	0.1972
3	6.6625	44.3889	16	0.0717

Source	SS	df	MS	F
Between	[illegible]	[illegible]	2.4230	18.44
Within	[illegible]	[illegible]	0.1314	
Total	[illegible]			

Reject hypothesis of no inoculation effect.

Multiple comparisons

$\sqrt{MSE} = \sqrt{0.1314} = 0.363$

Case (a) $S\bar{d} = 0.327$ Total serum protein of rabbits inoculated with live cells differed significantly from those which received formalized cells.

Case (b) $S\bar{d} = 0.352$ A significant increase in the total serum protein was determined in the rabbits which received live cells of L. pomona. This increase was determined in comparison to those normal values.

Statistical Analysis of the Value of the Serum Protein Fractions

I. Albumin

$\sum_{i}\sum_{j} y_{ij} = 205.94$

$\sum_{i}\sum_{j} y_{ij}^2 = 971.5420$

$\bar{y} = \frac{y_{..}}{44} = 4.6805$

$\bar{y}^2 = 21.9071$

1	y_i	y_i^2	J_i	s_i^2
1	4.6775	21.8790	12	0.0774
2	4.8462	23.4857	16	0.2454
3	4.5419	20.6289	16	0.1500

Source	SS	df	MS	F
Between groups	4.4692	2	2.2346	28.99
Within groups	3.1604	41	0.07708	
Total	7.6296	43		

Since $F_{.05,\ 2,\ 41} = 3.23$ 28.99, reject hypothesis of no effect.

To find out where the effect took place:

Multiple Comparison:

$\sqrt{MSE} = \sqrt{0.077} = 0.278$

Case (a) $S\bar{\sigma} = 0.25$ Significant difference in the response of the two groups of inoculated animals was found.

Case (b) $S\bar{\sigma} = 0.27$ No significant increase in the albumin was determined in either group of inoculated animals when compared to the normal values.

II. α-globulin

$\sum_i \sum_j y_{ij} = 17.$[illegible]

$\bar{y} = 0.3$[illegible]

$\sum_i \sum_j y_{ij}^2 = 7.4249$

$\bar{y}^2 = 0.1512$

i	y_i	y_i^2	J_i	S_i^2
1	0.2675	0.0716	12	0.00285
2	0.4838	0.2389	16	0.0208
3	0.3800	0.1444	16	0.00604

Source	SS	df	MS	F
Between groups	0.3392	2	0.1696	16.00
Within groups	0.4329	41	0.0106	
Total	0.7721			

Reject hypothesis of no effect.

Multiple Comparison

$\sqrt{MSE} = 0.103$

Case (a) $Se^{\wedge} = 0.0926$ There was a significant difference in the response of the two groups of inoculated animals.

Case (b) $Se^{\wedge} = 0.10$ A significant increase in the α-globulin was determined in both groups of inoculated animals, when statistically compared to normal values.

III. β-globulin

$\sum_i \sum_j y_{ij} = 29.39$ $\sum_i \sum_j y_{ij}^2 = 20.4773$

$\bar{\mu} = 0.6680$ $\bar{\mu}^2 = 0.4462$

Source	SS	df	MS	F
Between groups	0.3076	2	0.1538	10.91
Within groups	0.5769	41	0.0141	
Total	0.8845	43		

Reject hypothesis of no effect.

Multiple Comparison

$\sqrt{MSE} = 0.119$

Case (a) $S\hat{\sigma} = 0.107$ There was a significant difference in the response of the two groups of inoculated animals.

Case (b) $S\hat{\sigma} = 0.115$ A significant increase in β-globulin was found only in those animals which received live cells of *L. pomona*.

IV. γ-globulin

$\sum_i \sum_j y_{ij} = 22.55$ $\sum_i \sum_j y_{ij}^2 = 13.7623$

i	y_i	y_i^2	J_i	S_i^2
1	0.4183	0.1750	12	0.0067
2	0.5894	0.3474	16	0.0452
3	0.5062	0.2562	16	0.0833

Source	SS	df	MS	F
Between groups	0.1988	2	0.0994	2.03
Within groups	2.0047	41	0.0489	
Total	2.2035	43		

Do not reject hypothesis of no effect.

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BIOGRAPHICAL SKETCH

Suhaila Saadallah was born March 7, 1939, at Mosul, Iraq. In June, 1959, she received the degree of Bachelor of Science with a major in Microbiology from Baghdad University. From September, 1959, to August, 1963, she was employed as a Laboratory Instructor in the Department of Bacteriology, Medical School, Baghdad University. From September, 1963, to August, 1965, she was a graduate student in the Department of Bacteriology at the University of Florida and received the degree of Master of Science in August, 1965. Since that time she has continued graduate studies in the Department of Bacteriology and is now a candidate for the degree of Doctor of Philosophy.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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